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Biologie de la reproduction, diversité génétique et spatiale de deux espèces du genre *Vanilla* (Orchidaceae) du sud-ouest de l'océan Indien : *V. humblotii* et *V. roscheri*. Implications pour leur conservation

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Арара...

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Ce manuscrit de thèse est présenté sous forme d'articles de recherche publiés ou en cours de soumission.

Chapitre d'ouvrage

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Gigant R, De Bruyn A, Guiot V, Viscardi G, Gigord L, Gauvin-Bialecki A, Pailler T, Humeau L, Grisoni M, Besse P (in prep) Reproductive strategies and their consequences on fine-scale spatial genetic structure and diversity of the indigenous and endangered leafless *Vanilla humblotii* (Orchidaceae) from Mayotte (Comoros Archipelago, Indian Ocean).

Article 3

Gigant R, De Bruyn A, Guiot V, Viscardi G, Gigord L, Humeau L, Chevalier MH, Grisoni M, Besse P (in prep) Impact of fragmentation on the genetic diversity of *Vanilla humblotii* Rchb. f. (Orchidaceae) in Mayotte (Comoros Archipelago, Indian Ocean) and implications for conservation.

Article 4

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CHAPITRE 1

CHAPITRE 1 : Introduction

1.1 Introduction générale

La conservation de la biodiversité est devenue un enjeu majeur au cours des dernières décennies au regard des grands bouleversements environnementaux de notre planète. Ceux-ci sont principalement liés à la destruction des habitats naturels, à l'introduction d'espèces exotiques, à la surexploitation de certaines espèces et aux pollutions induites partiellement sinon entièrement par l'homme. La définition, par les biologistes, des priorités et des méthodes de la conservation a longtemps été sujet à débat, notamment entre écologues et généticiens. Pour évaluer la survie des espèces, les uns privilégiaient davantage les facteurs démographiques et environnementaux (Beissinger and Westphal 1998) par rapport aux niveaux de diversité et à la différentiation génétique des populations (Frankel 1974; Frankham 1995; Escudero et al. 2003). L'avènement des approches intégratives (biologie de la conservation, génétique des populations, génétique de la conservation, écologie moléculaire) a permis d'appréhender les différents niveaux d'organisation génétique, spécifique et écosystémique du vivant sous un nouvel angle qui rend compte maintenant de l'état d'interaction dynamique entre le vivant et l'environnement. En effet, la susceptibilité d'une population végétale fragmentée, souvent de taille réduite et isolée des autres populations, dépend aussi bien de facteurs écologiques tels que les effets de bords, les aléas démographiques ou climatiques ou les pertes d'interaction avec d'autres espèces cruciales (pollinisation/dispersion) (Higgins et al. 2000; Oostermeijer 2003; Lande 1993) que de facteurs génétiques, à savoir la perte de diversité, la dépression de consanguinité ou l'accumulation de mutations délétères (Lande 1994; Lande and Shannon 1996; Lande 1988, 1998; Ellstrand and Elam 1993; Frankham 2005, 1995; Lande 1995). Les méthodes d'analyses spatiales de la diversité génétique illustrent l'application combinée en biologie de la conservation des principes de l'écologie et de la génétique (Escudero et al. 2003; Peakall and Beattie 1996; Peakall and Beattie 1995; Chung et al. 1999; Debout et al. 2011; Hardy 1999; Mhemmed et al. 2008; Vekemans and Hardy 2004). La diversité génétique est effectivement

variable en fonction des niveaux d'organisation du vivant considéré dans son environnement, allant des individus voisins à la population d'individus jusqu'aux grands ensembles de populations, nécessairement le fruit de l'influence de l'environnement, des traits d'histoire de vie et du passé démographique de l'espèce (Loveless and Hamrick 1984; Slatkin 1985; Escudero et al. 2003).

Il est tout aussi important dans une approche consistant à caractériser la biodiversité, de comprendre son origine et les mécanismes présidant à son émergence. Pour cela, les études taxonomiques couplées aux méthodes de reconstruction phylogénétiques viennent compléter l'arsenal du biologiste. Le « DNA barcoding », qui consiste à réaliser le marquage moléculaire systématique du vivant à l'aide d'une courte séquence nucléotidique, en est un des exemples les plus en vogue (Kress et al. 2005; Hajibabaei et al. 2007; Kress et al. 2009; Cowan et al. 2006). Par le bais des séquences nucléotidiques, des espèces cryptiques vivant en sympatrie avec d'autres espèces, ont été identifiées en tant qu'entités taxonomiques (Lahaye et al. 2008; Bickford et al. 2007; Olsson et al. 2005; Gómez et al. 2002). Dans la famille des Orchidaceae, la phylogénie moléculaire a ainsi permis de clarifier la classification aussi bien à l'échelle générique (Bytebier et al. 2008; Bytebier et al. 2007; Bogarín and Pupulin 2010) qu'au niveau des grandes subdivisions. Cinq sous-familles monophylétiques sont maintenant reconnues (Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae, and Epidendroideae (Chase et al. 2003)).

Le genre *Vanilla* Plumier ex. Miller (Vanilloideae, Orchidaceae) permet de rendre compte à la fois de la richesse et de la complexité des relations entre espèces végétales. De par la prépondérance de la reproduction végétative (Portères 1954), l'existence d'hybridations interspécifiques (Bory et al. 2010; Bory et al. 2008c; Nielsen 2000; Nielsen and Siegismund 1999; Lubinsky et al. 2008; Minoo et al. 2006) et la caractérisation cytogénétique récente de morphes intraspécifiques de niveaux de ploïdie différents (Bory et al. 2010; Bory et al. 2008a; Lepers-Andrzejewski et al. 2011a; Lepers-Andrzejewski et al. 2011b), ce genre nous offre la possibilité d'étudier un « groupe taxonomique complexe » (TCG) tel que défini par Ennos et al. (2005) et revêt donc un intérêt tout particulier pour les biologistes de la conservation. Par ailleurs, la deuxième épice la plus chère sur le marché mondial après le safran

est aussi une orchidée du genre *Vanilla,* la seule orchidée cultivée pour son fruit : *Vanilla planifolia* G. Jackson*.* Le matériel végétal cultivé à travers le monde caractérisé à l'aide d'outils moléculaires par Bory et al. (2008c), est extrêmement uniforme, ce qui renforce l'importance de la conservation des espèces du genre *Vanilla* (Grisoni et al. 2006). Pernès et al. (1984) ont déjà souligné l'intérêt d'étudier les complexes d'espèces, à la fois du point de vue de la génétique des populations comme de la macroévolution. En s'associant pour caractériser conjointement les barrières aux échanges géniques, le sélectionneur et le biologiste de la conservation tirent ainsi partie de la diversification des espèces qui s'est jouée durant plusieurs millions d'années d'évolution et sélection naturelle.

Face aux menaces émergentes, d'ordre biotique (maladies fongiques et virales) (Odoux and Grisoni 2011; Farreyrol 2005; Farreyrol et al. 2001; Grisoni et al. 2004) et abiotique (aridification des zones tropicales) (Le Treut and Jancovici 2004) qui pèsent sur le maintien de la production mondiale de vanille, il est nécessaire de préserver et d'étudier le germplasm du genre *Vanilla.* Celui-ci peut en effet être source de variabilité génétique ainsi que de caractères d'amélioration de l'espèce cultivée. L'existence d'hybrides interspécifiques naturels dans le genre a été démontrée (Nielsen 2000; Nielsen and Siegismund 1999; Lubinsky et al. 2008; Bory et al. 2008c). De plus, dans les années 1940-1960, des programmes de croisements interspécifiques ont été réalisés avec succès à Madagascar, aux Comores et à Puerto Rico (Bourriquet 1954; Childers and Cibes 1948) et plus récemment en Inde (Minoo et al. 2006). Ces divers travaux montrent que le genre *Vanilla* peut être considéré comme un complexe d'espèces potentiellement interfertiles puisque des espèces phylogénétiquement fort éloignées, telles *V. planifolia* (groupe américain aromatique) et *V. aphylla* Blume (groupe afro-asiatique) ont pu être croisées et donner une descendance (Minoo et al. 2006). Ces données démontrent qu'il est envisageable d'exploiter le germplasm du genre *Vanilla* à des fins d'amélioration variétale.

La conservation de ce germplasm revêt d'autant plus d'importance que l'habitat naturel des vanilliers est menacé sur les trois continents américain, africain et asiatique où le genre est présent (Bory et al. 2008b; Soto Arenas and Cameron 2003; Portères 1954). L'Amérique tropicale, qui est aussi la région

d'origine du genre *Vanilla* (Cameron 2000; Bouetard et al. 2010), porte à elle seule près de la moitié de la diversité (52 espèces) des vanilliers (Portères 1954) et héberge les 18 à 35 espèces à fruit aromatique (Soto Arenas and Cameron 2003). Il s'agit d'une région particulièrement menacée (Laurance et al. 2000), où les conséquences de la surexploitation de certaines espèces ont déjà entrainé la perte de dizaines de millions d'hectares de leur distribution originelle (André et al. 2008; Grogan et al. 2010). Ceci est d'autant plus alarmant que les simulations basées sur l'évolution du climat prévoient une réduction de 18% des forêts tropicales en Amérique du Sud dans moins d'un siècle (Salazar et al. 2007).

Par ailleurs, certains vanilliers montrent une adaptation particulière aux conditions environnementales xériques. Il s'agit des vanilliers de la Section Aphyllae (Rolfe 1896) dont les feuilles sont très petites, souvent réduites à des écailles. Ces vanilliers sont bien adaptés aux forêts tropicales sèches des régions insulaires et littorales d'Afrique, d'Asie et des Caraïbes. A travers le monde, les forêts tropicales sèches sont considérées comme les écosystèmes tropicaux forestiers les plus menacés (Janzen 1988; Miles et al. 2006; Hoekstra et al. 2005). Les menaces diffèrent selon le continent : liées majoritairement au changement climatique et à la transformation des territoires pour l'agriculture sur le continent américain (Gasparri and Grau 2009; Grau et al. 2005; Boletta et al. 2006; Peralvo et al. 2007; Quesada et al. 2009; Quesada and Stoner 2004), à la fragmentation de l'habitat et aux feux de forêts en Afrique et à Madagascar où des mesures de conservation sont insuffisantes (Miles et al. 2006; Elmqvist et al. 2007) et aux densités de populations en Eurasie (Miles et al. 2006). La conservation des vanilliers aphylles est d'autant plus une priorité que ces vanilliers peuvent être des sources d'amélioration des espèces cultivées, de par leur adaptation à la sécheresse et leur rusticité.

C'est dans ce cadre général qu'a été mise en place dès 2003 au sein du laboratoire UMR PVBMT (Peuplements Végétaux et Bioagresseurs en Milieu Tropical) à la Réunion, une collection *ex situ* de ressources génétiques de vanilliers. Cette collection, récemment labélisée Centre de Ressource Biologique (CRB) Vatel, rassemble plus de 1000 accessions et couvre une trentaine d'espèces (Grisoni

et al. 2007; Roux-Cuvelier and Grisoni 2010). Certaines espèces ont d'ores et déjà été identifiées comme sources potentielles de caractères d'intérêts agronomiques, tels que l'autofertilité chez *V. lindmaniana* Kraenzl. et *V. palmarum* (Salzm. ex Lindl.) Lindl., la résistance à la fusariose (maladie fongique) chez *V. pompona* Schiede et *V. bahiana* Hoehne, l'indéhiscence des fruits chez *V. tahitensis* J. W. Moore et certaines accessions de *V. planifolia.*

Ce travail de thèse s'inscrit dans la volonté d'améliorer et d'optimiser la conservation *in situ* et *ex situ* des vanilliers. Il s'appuie sur des études fondamentales concernant l'écologie et la génétique des espèces pour déterminer la biologie de la reproduction, la variabilité génétique, dans la mesure du possible à différentes échelles spatiales, chez deux vanilliers sauvages aphylles de l'océan Indien *V. humblotii* Rchb. f. à Mayotte et *V. roscheri* Rchb. f. en Afrique Du Sud.

En préalable, une synthèse bibliographique est proposée sous forme d'un chapitre d'ouvrage (*Biodiversity and Evolution in the* Vanilla *Genus* publié dans *The Dynamical Processes of Biodiversity; Case Studies of Evolution and Spatial Distribution*, édité par O. Grillo et G. Venora) afin de mieux appréhender l'état actuel de nos connaissances sur la biodiversité et l'évolution du genre *Vanilla.* Les relations interspécifiques et la reproduction des espèces sauvages en conditions naturelles ainsi que l'évolution des espèces en conditions cultivées y sont particulièrement détaillés. Le cas particulier des espèces aphylles du genre présentes dans l'océan Indien est ensuite abordé, afin de présenter les connaissances actuellement disponibles et les menaces anthropiques pesant sur ces espèces qui nécessitent des mesures urgentes de conservation.

1

Biodiversity and Evolution in the *Vanilla* Genus

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1. Introduction

Since the publication of the first vanilla book by Bouriquet (1954c) and the more recent review on vanilla biodiversity (Bory et al., 2008b), there has been a world regain of interest for this genus, as witnessed by the recently published vanilla books (Cameron, 2011a; Havkin-Frenkel & Belanger, 2011; Odoux & Grisoni, 2010). A large amount of new data regarding the genus biodiversity and its evolution has also been obtained. These will be reviewed in the present paper and new data will also be presented.

2. Biogeography, taxonomy and phylogeny

2.1 Distribution and phylogeography

Vanilla Plum. ex Miller is an ancient genus in the Orchidaceae family, Vanilloideae sub-family, Vanilleae tribe and Vanillinae sub-tribe (Cameron, 2004, 2005).

Vanilla species are distributed throughout the tropics between the 27th north and south parallels, but are absent in Australia. The genus is most diverse in tropical America (52 species), and can also be found in Africa (14 species) and the Indian ocean islands (10 species), South-East Asia and New Guinea (31 species) and Pacific islands (3 species) (Portères, 1954). From floral morphological observations, Portères (1954) suggested a primary diversification centre of the *Vanilla* genus in Indo-Malaysia, followed by dispersion on one hand from Asia to Pacific and then America, and on the other hand from Madagascar to Africa. This hypothesis was rejected following the first phylogenetic studies of the genus (Cameron, 1999, 2000) which suggested a different scenario with an American origin of the genus (160 to 120 Mya) and a transcontinental migration of the *Vanilla* genus before the break-up of Gondwana (Cameron, 2000, 2003, 2005; Cameron et al., 1999). The genetic differentiation between New World and Old World species observed would therefore be a consequence of the further separation of the continents. Our recent molecular phylogeny using chloroplastic *psa*B, *psb*B, *ps*bC, and *rbc*L regions (Bouetard et al., 2010) supported the hypothesis of an American origin of the genus (figure 1). However, the recent discovery of a fossilized orchid pollinaria (20 Mya) (Ramirez et al., 2007) allowed the dating of Vanilloidae sub family at 72 Mya, well after the separation of Gondwana which questions the hypothesis of a vicariate evolution of the *Vanilla* genus (Bouetard et al., 2010).

Transoceanic dispersion appears more credible and would have been implied at least three times in the evolution of the *Vanilla* genus (figure 1). This was demonstrated by dating a *Vanilla* molecular phylogeny, testing these two extreme evolutionary scenarios (vicariate

versus transoceanic dispersion) (Bouetard et al., 2010) (figure 1). The Gondwanan dispersion scenario used 95 Mya as prior on the NW/OW node (the minimum age assumption for the break-up of Gondwana), whereas the NW/OW transoceanic dispersion scenario used 71 Mya as prior on the Vanilloidae node (a date estimated from fossil orchid pollinaria dating (Ramirez et al., 2007)) (figure 1). This provided evidence for at least three transoceanic dispersion events whatever the original scenario retained for the differentiation of NW versus OW species: from Africa to Asia, from Africa to the South West Indian Ocean Islands, and from Africa back to America (Carribean region) (Bouetard et al., 2010) (figure 1).

2.2 Taxonomy and phylogeny

Taxonomic classification is based on morphological variations in vegetative and floral characters. Ephemeral flowers and their scarce availability in herbarium specimens associated with the fact that vegetative characters show important intra-specific variations are responsible for the difficulties in providing a clear taxonomic classification in *Vanilla* (Bory et al., 2010).

The first classification (Rolfe, 1896) distinguished two sections in the genus: section Foliosae, and section Aphyllae with leafy or leafless species, respectively. Portères (1954) then divided section Foliosae in three sub-sections: Papillosae, with thick leaves and a labellum with fleshy hairs, Lamellosae with thick leaves and a labellum with scaly lamellae, and Membranacae with thin membranous leaves.

The *Vanilla* genus taxonomy has recently greatly beneficiated from molecular phylogenetics. The sequences used were chloroplastic *rcb*L (Cameron et al., 1999; Soto Arenas & Cameron, 2003), *psa*B (Cameron, 2004), *psb*B and *psb*C (Cameron & Molina, 2006), and the results obtained showed that Rolfe's sections and Portères' sub-sections classically used for taxonomy in *Vanilla* did not have a phylogenetic value. A recent study (Bouetard et al., 2010), based on these four markers combined, revealed three major clades in the genus, called groups α, β, et γ (figure 1). Group α is represented by *V. mexicana* and is ancestral. Separation between group β (composed of New World/American Foliosae species) and group γ (composed of Old World/African and Asian Foliosae and American, Asian and African Aphyllae species) is more recent. This study confirmed an American origin of the genus, and also showed that the sections Foliosae and Aphyllae are not monophyletic (figure 1), a statement that questions the classical taxonomic treatment of the genus proposed by Rolfe (1896) and Portères (1954).

Recently, based on phylogenetic data of 106 species, (Soto Arenas & Cribb, 2010) proposed a new taxonomic classification, differentiating two sub-genera in the *Vanilla* genus. A group contains species previously classified as sub-section Membranaceae: *V. angustipetala*, *V. martinezii*, *V. inodora*, *V. mexicana*, *V. parviflora*, *V. edwalii* and the monospecific genus *Dictyophyllaria dietschiana* now *V. dietschiana* (Bouetard et al., 2010; Cameron, 2010; Pansarin, 2010a2010b; Soto Arenas & Cameron, 2003). It was named genus *Vanilla* sub-genus *Vanilla* as it contains the typus species for the genus (*V. mexicana*). It corresponds to the ancestral phylogenetic group α (figure 1). The remaining *Vanilla* species are included in genus *Vanilla* sub-genus *Xanata*, which is further divided in two sections: section *Xanata* (corresponding to phylogenetic group β) and section *Tethya* (group γ) (figure 1). Within section *Xanata*, an early diverging group is noteworthy (figure 1) containing *V. palmarum*, *V. lindmaniana* and *V. bicolor* (Bouetard et al., 2010; Cameron, 2010; Soto Arenas & Cameron, 2003). This preliminary revised classification is a major step towards a needed complete revision of the genus based on molecular analyses.

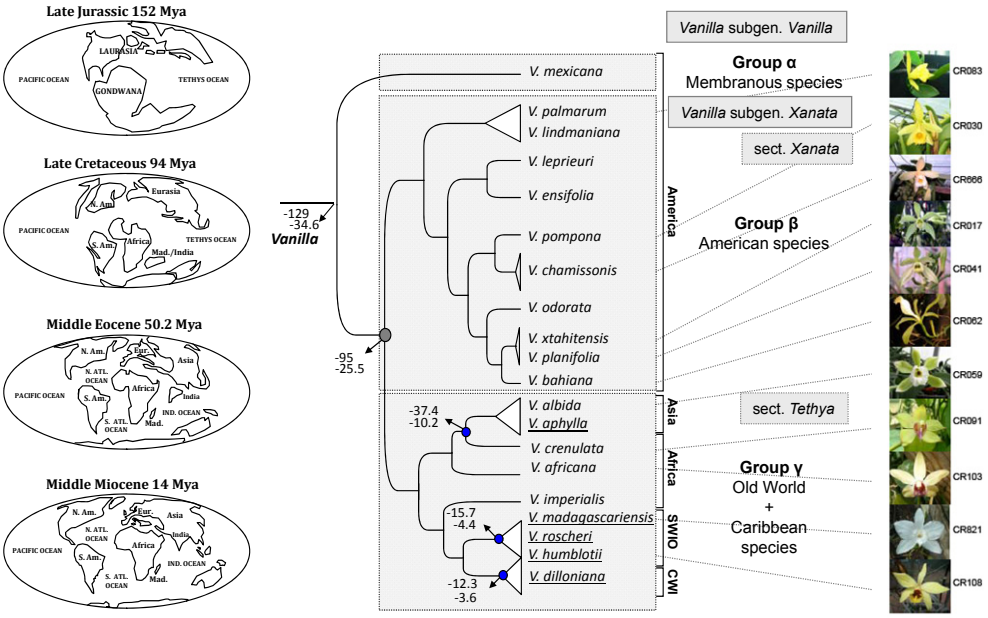

Fig. 1. Schematic representation of the molecular phylogeny of the *Vanilla* genus based on *rbc*L, *psa*B, *psb*B and *psb*C (Bouetard et al., 2010), distinguishing clades α, β and γ. The geographical origin of the species is indicated. Species underlined are from sect. Aphyllae, others are from sect. Foliosae (as per Rolfe's classification). Taxonomic classification as per Soto Arenas & Dressler (2010) is indicated. Flowers of representative species and their voucher number (CR) in the BRC Vatel collection are presented (photographs: M Grisoni). Estimated divergence times (in Mya) derived from Bayesian relaxed clock analyses (uncorrelated exponential relaxed molecular clock model) (Bouetard et al., 2010) are indicated for key nodes: (i) origin of *Vanilla*, (ii) separation between New and Old World *Vanilla* species; (iii) separation between African and Asian species; origin of Aphyllae species (iv) in the South West Indian Ocean area and (v) in the Caribbean-West Indies area. Upper values correspond to the Gondwanan dispersion scenario and lower values correspond to the transoceanic dispersion scenario. Blue dots on clade nodes indicate transoceanic dispersion whatever the scenario tested. World maps at different geological times are provided.

In the first thorough taxonomic treatment of the genus published, Portères (Portères, 1954) described 110 species in the *Vanilla* genus. This number was reduced by different authors (Cameron et al., 1999; Soto Arenas, 1999, 2006; Soto Arenas & Dressler, 2010), but some species were not included (Hoehne, 1945) and new species have since been described (Z.J. Liu et al., 2007; Pignal, 1994; Soto Arenas, 2006, 2010; Soto Arenas & Cameron, 2003; Szlachetko & Veyret, 1995). There are to date more than 200 *Vanilla* species described (Bory et al., 2008b; Cameron, 2011b), but numerous synonymies remain and there is therefore an urgent need to thoroughly revise the taxonomic classification of the *Vanilla* species. We recently reviewed (Bory et al., 2010) the complexity of the processes involved in the evolution and diversification

of the *Vanilla* genus and concluded that *Vanilla* must be considered as a TCG, a "Taxonomic Complex Group" (Ennos et al., 2005). Indeed, it exhibits (i) an uniparental reproduction mode (vegetative growth) (Portères, 1954) (ii) interspecific hybridization in sympatric areas (Bory et al., 2010; Bory et al., 2008c; Nielsen, 2000; Nielsen & Siegismund, 1999) and (iii) polyploidy (Bory et al., 2010; Bory et al., 2008a; Lepers-Andrzejewski et al., 2011a; Lepers-Andrzejewski et al., 2011b). These mechanisms have profound effects on the organization of the biological diversity and have been described as responsible for the difficulty to define discrete, stable and coherent taxa in such TCGs (Ennos et al., 2005). *Vanilla* is a typical example of a genus for which the barcoding protocols (*mat*K and *rbc*L) as proposed by the CBOL (M.L. Hollingsworth et al., 2009; P.M. Hollingsworth & CBOL Plant Working Group, 2009 ; Ratnasingham & Hebert, 2007), will therefore not be sufficient to revise the species taxonomy. The lack of genetic incompatibility between most *Vanilla* species (Bory et al., 2010) and the proven occurrence of inter-specific hybridizations in the genus (Bory et al., 2010; Bory et al., 2008c; Nielsen, 2000; Nielsen & Siegismund, 1999) will necessitate the obligate survey of nuclear regions in addition to cpDNA markers to resolve introgression patterns and correctly identify *Vanilla* species (Rubinoff, 2006). As an example, the species *V.* ×*tahitensis* was recently shown to be a *V. planifolia* x *V. odorata* hybrid using a combined ITS and chloroplastic phylogenetic analysis (Lubinsky et al., 2008b), when chloroplastic DNA alone repeatedly identified this species as identical to its maternal donor parent *V. planifolia* (figure 1). Moreover molecular genetic diagnostics can only be useful for barcoding biodiversity when species delimitations are either subtle or cryptic but nonetheless clear-cut. In a TCG, taxon limits are themselves diffuse, therefore genetic analysis alone might fail in the identification of discrete species (Ennos et al., 2005). A typical example of expected difficulties will be within the *V. pompona* species complex which was recently described as containing subspecies *pompona*, *pittieri*, and *grandiflora* based on ITS data, although the latter two are rather paraphyletic (Soto Arenas & Cribb, 2010) . In *Vanilla*, taxonomic revision of species will therefore have to use a combination of taxonomic, morphological, ecological, reproductive biology, cytogenetic (polyploidy estimates) and genetic (nuclear and chloroplastic) assessments.

3. *Vanilla* biodiversity in the wild

Most *Vanilla* species are hemiepiphytic vines climbing up to 30 meters high (*V. insignis*) (Soto Arenas & Dressler, 2010) and growing in tropical wet forests between 0-1000m (Portères, 1954). Only a few species are adapted to drier conditions (*V. calycullata*, (Soto Arenas & Dressler, 2010)), although extreme xeric adaptation is observed in the 18 leafless species of the genus (Portères, 1954). Vegetative reproduction (by natural stem cuttings) is the predominant reproduction mode adopted by most *Vanilla* species to develop settlements, such as *V. bahiana*, *V. chamissonis*, *V. madagascariensis*, *V. dilloniana*, *V. barbellata*, *V. claviculata* (reviewed in (Bory et al., 2010)). Some vines can grow up to 100 meters long (*V. insignis* (Soto Arenas & Dressler, 2010)) and in *V. planifolia* the same individual can cover up to 0.2ha (Soto Arenas, 1999). However a few species might be strictly sexually reproducing, such as *V. bicolor* and *V. palmarum* which are described as epiphytic on palm trees (Householder et al., 2010; Pignal, 1994), and *V. mexicana* (Bory et al., 2010; Cameron, 2010). Another notable exception is the species *V. dietschiana* which is non lianescent and 40 cm high, and has long been classified for these reasons as a different genus *Dictyophyllaria* (Pansarin, 2010a, 2010b; Portères, 1954).

In natural conditions, vanilla plant density can be extremely variable from being very high in certain areas (*V. trigonocarpa* (Soto Arenas & Dressler, 2010), *V. pompona* (Householder et al., 2010)) from very low as reported for wild *V. planifolia* in Mexico with less than one plant found per square kilometre (Soto Arenas, 1999). Some species are known to flower very frequently (*V. chamissonis,* (Macedo Reis, 2000)) to very un-frequently (*V. planifolia, V. hartii*, (Schlüter, 2002; Soto Arenas & Dressler, 2010)). A single flower per inflorescence generally opens in *Vanilla*, except 2-3 in some species (*V. odorata, V. martinezii, V. insignis*) and flowers are ephemeral (one day) except for some rare species such as *V. inodora* (2-3 days) (Soto Arenas & Dressler, 2010) or *V. imperialis* for which the flowers can be fertilized 4-5 days after opening (unpublished data). Seedlings can be found very frequently for species such as *V. bicolor* and *V. palmarum* (Householder et al., 2010) or be extremely rare as in *V. pompona* in Madre de Dios (Householder et al., 2010) or *V. planifolia* in Mexico (Schlüter, 2002). All these natural history traits will have deep effects on the levels of *Vanilla* species biodiversity that can be found in the wild. Particularly, the relative balance between vegetative and sexual reproduction and their relative efficiency will be of major importance in shaping populations genetic diversity. Exploring *Vanilla* species reproductive systems is therefore essential in this context.

3.1 *Vanilla* pollination

Vanilla species, like other orchids, are characterized by the presence of a rostellum membrane separating female and male reproductive systems, therefore limiting self-pollination. The diverse floral morphology observed in *Vanilla* species (figure 1) suggests that they have evolved to adapt to different pollinators (Soto Arenas & Cameron, 2003).

3.1.1 Self-pollinating species

A few *Vanilla* species are described as spontaneously self-pollinating (Householder et al., 2010; Soto Arenas & Cameron, 2003; Soto Arenas & Dressler, 2010; Van Dam et al., 2010), as suggested by their abnormally high fruit set (table 1). This is consistent with general data in orchids showing that autogamous species display a much higher fruit set (77%) than cross pollinating species for which the majority show fruit set <20% (Tremblay et al., 2005). Based on high fruit set, these suggested autogamous species are *V. palmarum*, *V. savannarum*, *V. bicolor* (American species of the *V. palmarum* group), *V. guianensis*, *V. martinezii* (American species of the *V. mexicana* group) and *V. griffithii* (an Asian species). Possible self-pollination for *V. inodora* is also reported (Soto Arenas & Dressler, 2010), due to the large fruit set observed in some populations, although others have a fruit set as low as 2.5%.

Species	Natural fruit set (self-pollination)	Reference
V guianensis	78%	(Householder et al., 2010)
V. palmarum	76%	(Householder et al., 2010)
V bicolor	71%	(Householder et al., 2010)
V. bicolor	42.5% per raceme	(Van Dam et al., 2010)
V. martinezii	53% in a clone	(Soto Arenas & Dressler, 2010)

Table 1. Suggested self-pollinating *Vanilla* species and recorded natural fruit sets.

More precise observations are available for some of these species. *V. guianensis* is supposedly self-pollinated at early anthesis, as it was observed that the stigma and the

anther grew to contact one another; and no pollinators were observed despite the high fruit set recorded in Peru (Householder et al., 2010). The lack of observed local pollinators and the high fruit set also suggested that *V. bicolor* and *V. palmarum* were self-pollinating species in Peru (Householder et al., 2010).

Two mechanisms were proposed to account for self-pollination in *Vanilla* species (Van Dam et al., 2010): true self-pollination occurring by either stigmatic leak and/or the presence of a dehydrated or reduced rostellum, or agamospermy. In *V. bicolor*, pollen removal experiments showed that agamospermy was not the mechanism in play (Van Dam et al., 2010). Also all fertilized flowers showed fully developed rostellum. This suggested that a stigmatic leak, where stigma lobes release a fluid that contacts the pollen and induces germination of the pollen tubes (Van Der Pijl & Dodson, 1966) was the more likely explanation for self-pollination in this species (Van Dam et al., 2010). The observation of the occurrence of a thick rostellum in *V. palmarum* led to the suggestion of an identical mechanism (Householder et al., 2010). Our own observations on *V. palmarum* reveal self-pollination most likely due to a rostellum reduced in width, allowing pollinaria to get in contact with the stigmata on both sides of the rostellum (figure 2). A similar situation is found for the self-fertile species *V. lindmaniana* (data not shown).

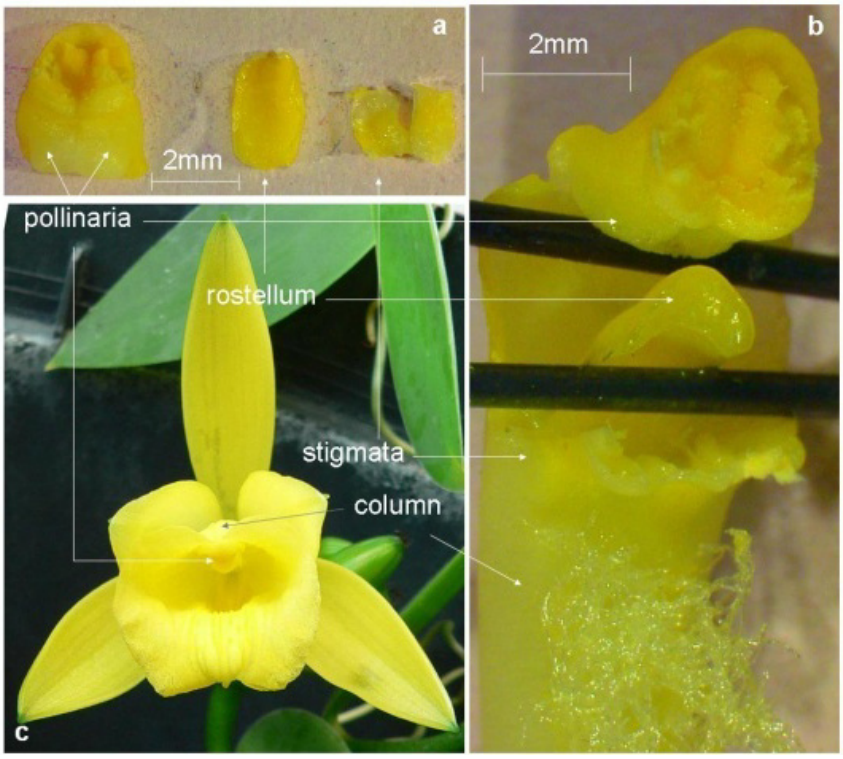

Fig. 2. Detailed structure of the pollinaria, rostellum and stigmata in the species *V. palmarum:* (a) and (b) accession CR0891, (c) accession CR0083, maintained in BRC Vatel (Reunion Island).

Spontaneous self-pollination is sometimes described even in classically outcrossing species. In Oaxaca plantations, cases of *V. planifolia* self-pollination are reported (Soto Arenas & Cameron, 2003) with rates reaching 6% of covered flowers giving fruit. Similar rates (6.06%) were reported for bagged *V. chamissonis* flowers in Sao Paulo (Macedo Reis, 2000). Nothing is known about the mechanisms involved in such exceptional cases.

3.1.2 Outcrossing species and pollinators

For the majority of *Vanilla* species, self-pollination does not occur due to an efficient rostellum and sexual reproduction therefore relies on the intervention of pollinators. Consequently, relatively low natural fruit sets are observed in natural conditions ((Bory et al., 2008b), table 2), consistent with the 17% median natural fruit set reported for tropical orchids (Tremblay et al., 2005). Reproductive success in orchids is pollination – rather than by resource - limited and could depend on pollinator effectiveness, abundance and diversity, and pollen quantity and quality (self *versus* allopollen) (Tremblay et al., 2005). This was demonstrated by crossing experiments in temperate and tropical orchids showing that cross hand-pollination shows significantly greater success (80%) than natural open pollination (26.6%) (Tremblay et al., 2005). Further studies are needed in *Vanilla* to determine the highest fruit sets achievable, but results on *V. barbellata, V. claviculata, V.dilloniana*, and *V. poitaei* have showed up to 100% fruit set under hand pollination experiments (Tremblay et al., 2005), and 75.76% in *V. chamissonis* (Macedo Reis, 2000), much higher values than what can be observed in natural conditions (table 2).

Species	Natural fruit set (open pollination)	Reference
V. barbellata	18.2 %	(Tremblay et al., 2005)
V. chamissonis	15%	(Macedo Reis, 2000)
V. claviculata	17.9 %	(Tremblay et al., 2005)
V. crenulata	0%	Johansson 1974, as cited in (Soto Arenas & Cameron, 2003)
V. cristato-callosa	6.6%	(Householder et al., 2010)
V. dilloniana	14.5 %	(Tremblay et al., 2005)
V. planifolia	1% to 1‰	(Soto Arenas, 1999)
V. planifolia	1%	(Childers & Cibes, 1948)
V. planifolia	1%	(Tremblay et al., 2005)
V. planifolia	1 à 3%	(Weiss, 2002)
V. poitaei	6.4 %	(Tremblay et al., 2005)
V. pompona subsp. grandiflora	0.9%	(Householder et al., 2010)
V. riberoi	1.1%	(Householder et al., 2010)

Table 2. *Vanilla* out-crossing species and natural fruit sets recorded.

If the pollinator of *V. planifolia* was long been considered as a social bee from the *Melipona* genus, as reported by Deltiel (as cited in (Rolfe, 1896)) and then mentioned in (Bouriquet, 1954a, 1954b; Stehlé, 1954), these records are now admitted as doubtful (Soto Arenas & Cameron, 2003; Van Der Cingel, 2001) as the bee is too small to perform the necessary

pollination steps (Lubinsky et al., 2006; Soto Arenas & Cameron, 2003). Lubinsky (2006), during observations of *V. planifolia* in Oaxaca (Mexico) and *V. pompona subsp. grandiflora* in Peru, indeed noticed *Melipona* visits, but no pollen movement was recorded. In tropical America (Guadeloupe (Stehlé, 1952) and Mexico (Stehlé, 1954)), authors have also reported the intervention of *Trigona* bees for *Vanilla* pollination, but this has never been confirmed. In Puerto Rico, leafless *Vanilla* species might be pollinated by *Centris* bees (Soto Arenas & Cameron, 2003). Hummingbirds are considered as vanilla pollinators in tropical America (Bouriquet, 1954a1954b; Stehlé, 1954). Lubinsky (2006) did indeed observe occasional *V. planifolia* visits by hummingbirds in Oaxaca, but with no pollen movement. Finally some authors (Dobat & Peikert-Holle, 1985; Geiselman et al., 2004) have suggested that the species *V. chamissonis* could be pollinated by two species of bats, although this fact was recently questioned (Fleming et al., 2009).

It is much more likely that in the American tropics, *Vanilla* is pollinated by large euglossine bees, as suggested by Dressler (1981) and demonstrated by such bees caught with *Vanilla* species pollinaria (Ackerman, 1983; Roubik & Ackerman, 1987). The principal reward offered by orchid flowers is nectar (Dressler, 1993), the most common reward for pollination (Van Der Pijl & Dodson, 1966). No *Vanilla* species has been described as producing floral nectar to our knowledge. However, the pollinators that visit orchid flowers can also obtain a variety of rewards (Singer, 2003; Tremblay et al., 2005) including oil, floral fragrances and, occasionally, pollen or stigmatic exudates (Bembe, 2004).

From years of observations in Mexico, Soto Arenas (Soto Arenas, 1999; Soto Arenas & Cameron, 2003) suggested the existence of three pollination systems for American *Vanilla* species (Bory et al., 2008b).

The first system relies on fragrance collection on flowers by male bees of the *Euglossa* genus, and has been suggested to concern the species of the *V. pompona* group as well as *V. hameri*, *V. cribbiana*, and *V. dressleri* (Soto Arenas, 1999; Soto Arenas & Cameron, 2003; Soto Arenas & Dressler, 2010). In this 'male euglossine syndrome' (Williams & Whiten, 1983) also referred to as 'perfume flower syndrome' (Bembe, 2004), now well known in many non nectar producing orchid species, male bees are attracted solely by the flower fragrance, and rub the surface of the flower with special tarsal brushes to collect fragrance materials, and subsequently store them in swollen glandular tibiae of the rear legs (Dodson et al., 1969). This fragrant orchid- male euglossine bee relationship is often highly specific (Dodson et al., 1969; Williams & Whiten, 1983). Bees then supposedly use these fragrance compounds as precursors for their own sex pheromones (Williams & Whiten, 1983) or in a "spraying" (of the fluid substances from their mid tibial tufts by vibrating action of their hind wings) behaviour as part of their courtship displays (Bembe, 2004). No study has so far been conducted to analyze *Vanilla* species flower fragrance compounds diversity and their relationship with pollinator specificity. This could give great insights on *Vanilla* evolution and diversity. On the other hand no direct evidence has been provided with regards to this male euglossine scent collection behaviour in any *Vanilla* flowers so far. Pollination of *V. trigonocarpa* by male *Euglossa asarophora* in Panama was reported (Soto Arenas & Dressler, 2010), with no information regarding scent collection behaviour. Male *Eulaema meriana* was identified as a possible pollinator for the species *V. pompona* subsp. *grandiflora* in Peru following observations of visits accompanied by pollen movement, but no scent collection behaviour was observed (Lubinsky et al., 2006). Similarly, some particularly fragrant flowers of this species were shown to attract two species of euglossine bees, *Eul. meriana* and *Eug. imperialis* (Householder et al., 2010). Only *Eul. meriana* was observed pollinating flowers on

two occasions, but no floral fragrance collection was recorded (Householder et al., 2010). This does not so far therefore confirm the suggested male euglossine syndrome within the *V. pompona* group. Most species seem to be pollinated under a deceptive system, as also suggested for *V. planifolia*, *V. odorata*, *V. insignis* and *V. hartii*, with flower visits by either male or female bees and an absence of reward (Soto Arenas, 1999; Soto Arenas & Cameron, 2003). This particular pollination system, using different strategies to lure pollinators, is mainly encountered in orchids with a third of the species in this family supposedly using this pollination system (Jersakova et al., 2006; Schiestl, 2005; Singer, 2003; Tremblay et al., 2005), particularly low density species (Ackerman, 1986), as it is the case for *V. planifolia* (Bory et al., 2008b; Soto Arenas, 1999). Soto Arenas considers the bee *Eugl. viridissima*, and maybe bees from the *Eulaema* genus, to be the real pollinators of *V. planifolia* (Bory et al., 2008b; Soto Arenas & Dressler, 2010). These species (as well as *Exeretes*) were recorded as occasional visitors of *V. planifolia* in Oaxaca (Mexico) without pollen movement (Lubinsky et al., 2006). *V. cribbiana* is reported to be pollinated by an unidentified *Eulaema* bee, *V. hartii* flowers are visited by female *Euglossa* bees and *V. insignis* flowers by male bees of *Eul. polychroma* (Soto Arenas & Dressler, 2010). The true pollinators of *V. planifolia* and most allied species therefore remain to be elucidated.

The last system might imply strong and large carpenter bees (*Xylocopa* species) and would concern the species *V. inodora*. This was suggested based on the peculiar floral structure of this species and allied Membranaceae (Soto Arenas & Cameron, 2003) characterized by a frontally closed labellum (the column apex lying on the lip) which is similar to that of other orchid species pollinated by carpenter bees (Soto Arenas & Cameron, 2003). These bees were observed visiting *V. inodora* but no proof of true pollination has been provided so far (Soto Arenas & Cameron, 2003; Soto Arenas & Dressler, 2010). The only data available on *Vanilla* potential pollinators, although partial, is therefore from America. There is a considerable lack of knowledge of potential *Vanilla* pollinators in other geographical areas. In Africa, euglossine bees do not occur, but other large bees may be pollinators there (Van Der Cingel, 2001). Despite three years of observation of the species *V. crenulata* in Africa, no pollinator visit was recorded (Johansson, 1974, as cited in (Soto Arenas & Cameron, 2003)). Observations in Madagascar of occasional natural fruit set in the introduced species *V. planifolia*, were attributed locally to sunbirds of the *Cynniris* genus (so called 'Sohimanga") (Bouriquet, 1954a). Similarly, in Reunion Island, rare natural pollination events of the introduced *V. planifolia* may be linked to noticed visits by the bird *Zosterops* (Zosteropidae) (Bory et al., 2008b), an Angraecoid orchid pollinator there (Micheneau et al., 2006). These hypotheses have not been confirmed, and remain unlikely as flower structure in *Vanilla* is indicative more of a bee pollination system (Dressler, 1981). Finally, a large bee of the *Aegilopa* genus was recorded pollinating *V. cf. kaniensis* in Papua New Guinea (Soto Arenas & Cameron, 2003). Although fruits of *V. albida* and *V. aphylla* from Java were described and illustrated in 1832, the introduced species *V. planifolia* did not naturally set fruit there, showing the need for different pollinators (Arditti et al., 2009). No other information is available regarding *Vanilla* pollinators in Asia (Van Der Cingel, 2001). It will be important to assess whether *Vanilla* species with higher fruit set (table 2) are characterized by reward pollination mechanisms as it was demonstrated that rewarding orchids show significantly higher fruit set than deceptive ones (twice as much) (Tremblay et al., 2005). Reproductive success might also be related to the fragrance attractiveness of flowers, even in a deceptive system. Further insights on this matter could be obtained by characterising *Vanilla* species floral fragrance and colour as well as identifying their respective pollinators and behaviour.

Partial information is available (Soto Arenas & Dressler, 2010) for *V. planifolia* stating the presence of 1-2-dimethyl-cyclopentane, ethyl acetate,1-8-cineol and ocimene-trans, and for *V. insignis* possessing the same principal constituents although ocimene-trans is notoriously absent. 1-8-cineol is especially well known to be a strong attractant for euglossine bees (Soto Arenas & Dressler, 2010). Our own observations (unpublished data) show that the species *V. chamissonis* displays particularly strongly fragrant flowers (more than *V. planifolia*), this could explain why its fruit set is amongst the highest.

3.2 Myrmecology

An obvious interaction exists between *Vanilla* and ants, as also demonstrated for other orchid species (Peakall, 1994). Extrafloral nectar is produced in immature bud abscission layer in many *Vanilla* species such as *V. pompona, V. cristato-callosa* in Peru (Householder et al., 2010) and *V. planifolia* in Panama (Peakall, 1994) and ants were observed in these species feeding on sugary exudates. Ants were also reported visiting *V. planifolia* flowers in Oaxaca (Lubinsky et al., 2006), without pollination. *V. planifolia* also occasionally inhabits ant nests, and was also observed to support ant nests in its root mass (Peakall, 1994).

The benefit of the association is obvious for the ant (food and shelter), but the benefit (if any) for the *Vanilla* plant remains to be elucidated. In some orchid species, ants visiting extrafloral nectaries have been shown in some cases to protect them against herbivory or to be attractors to bird pollinators (Peakall, 1994). Close association between ant nests and orchids have also suggested a role of ants in seed dispersion particularly in orchids with oily seeds (Peakall, 1994). In fragrant *Vanilla* fruits, seeds are held in an oily matrix (Householder et al., 2010). Ants have been reported in vanilla crop to be important for humus disintegration (Stehlé, 1954). On the other hand, the presence of ants could simply be indicative of the presence of mealybugs, softscales or aphids rather than an indication of a mutualistic interaction (Chuo et al., 1994). In *V. planifolia*, associations between scale and the black ant *Technomyrmex albipes* in Seychelles, as well as between ants and the aphid *Cerataphis lataniae* have been reported (Risbec, 1954).

3.3 Fragrance and bees and fruit dispersion

Seed dispersal mechanism(s) of *Vanilla* remains enigmatic. Fruits reaching maturity in many *Vanilla* species show dehiscence (Bouriquet, 1954c). This character favours seed dispersal, although it is noticeably not interesting in fruit crop production. In aromatic fruits, *Vanilla* seeds are easily rubbed off and are extremely sticky due to a thin covering of oil, which may favour epizoochorous seed dispersal by any visitor, insect or vertebrate (Householder et al., 2010). Soto Arenas and Cameron (2003) mentioned that *Vanilla* species producing fragrant fruits are restricted to tropical America and proposed the designation of group β (figure 1) as the 'American fragrant species' group, but this should not include species from the *V. palmarum* group as these were described as non-fragrant ((Householder et al., 2010), see below). Fruit fragrance was described as a pleisiomorphic character in orchids as it is present in *Vanilla* and in three other primitive groups (*Cyrtosia*, *Neuwiedia*, *Selenipedium*) (Lubinsky et al., 2006).

It has been demonstrated that euglossine bees are attracted by fragrant *Vanilla* fruits and act as seed collectors and potential dispersers. Van Dam et al. (2010) have photographed male *Eul. cingulata* with a typical scent collection behaviour on *V. pompona* subsp.*grandiflora* fruits in Peru. Householder et al. (2010) also reported strong attractiveness of fruit of this species

to *Eul. meriana* and *Eug. imperiali* which may stay on the same fruit for 15 minutes displaying typical scent collection behaviour. They also observed a similar behaviour by a metallic green *Euglossa* sp. on old and dehiscent *V. cristato-callosa* fruits. This confirmed previous observations of euglossine bees brushing on *Vanilla* fruits (Madison, 1981) and demonstrated the particular attractiveness of these bees to fragrant *Vanilla* flowers as well as to fragrant fruits, an important evolutionary step in the orchid/orchid-bee relationship in *Vanilla*. As discussed by Lubinsky et al. (2006), this demonstrates that the orchid/orchid-bee relationship has evolved in *Vanilla* as a mode of flower pollination as well as fruit dispersion *Trigona* bees were observed in Peru transporting sticky *V. pompona* seed packets on their hind tibia and often dropping them (Householder et al., 2010). These bees are not typical scent collectors and could just be interested in the nutritional value of the oils (Householder et al., 2010). One species of carpenter bee (*Xylocopa* sp) is also mentioned visiting *V. pompona* fruits (Householder et al., 2010).

Fruit dispersal by bats was suggested for *V. insignis* and observed for *V. pompona* (Soto Arenas & Dressler, 2010). Occasional total or partial herbivory of the fruit was also noticed for *V. pompona* in Peru, possibly attributed to bats or marsupials (Householder et al., 2010).

Bird dispersal is expected in some Asian species, as *V. abundiflora* and *V. griffithii*, as in the closely related Vanilloideae *Cyrtosia* genus (Soto Arenas & Dressler, 2010). However *Cyrtosia* has fleshy fruits like *Vanilla* but these are bright red presumably acting as an attractor to birds or mammals (Cameron, 2011b)

For some other *Vanilla* species however, fruits are non fragrant and seeds are not held in a particularly oily matrix. This is the case for *V. bicolor* and *V. palmarum* (Householder et al., 2010). Dehiscence of the fruits and canopy habitat suggested a different mechanism of seed dispersal in such species, by a combination of wind turbulence and gravity (Householder et al., 2010).

3.4 Conclusions

Many *Vanilla* species are threatened in the wild. This is particularly the case for *V. planifolia* in Mexico, its centre of origin. Proper conservation strategies need to be developed, but this will require gaining a better knowledge on the reproductive strategies and the derived levels of genetic diversity in these *Vanilla* species. This will include assessing the relative contribution of vegetative *vs* sexual reproduction, self-compatibility (auto *vs* allo fecundation success), pollination syndromes (pollinators, reward/deceit) and seed dispersion systems.

There is a considerable lack of genetic studies of *Vanilla* species biodiversity in the wild. The only published data concern the aphyllous species *V. barbellata*, *V. dilloniana* and *V. claviculata* on the island of Puerto Rico (Nielsen, 2000; Nielsen & Siegismund, 1999) using isozyme markers. Genotypic frequencies were in accordance with Hardy-Weinberg proportions for all species, which could suggest random crosspollination. High differentiation among populations was detected, supposedly attributed to limited seed dispersal by bees. Genetic drift was also demonstrated in some isolated populations (Nielsen & Siegismund, 1999). Soto Arenas also conducted *V. planifolia* population genetic studies in Mexico using isozymes (Soto Arenas, 1999), surprisingly demonstrating homozygous excess corresponding to preferential autogamous reproduction for this species.

Development of suitable approaches to the analysis of genetic diversity in a spatial context, where factors such as pollination, seed dispersal, breeding system, habitat heterogeneity and human influence are appropriately integrated in combination with molecular

population genetic estimates, will be essential (Escuderoa et al., 2003) to provide new insights in the understanding of the mechanisms of maintenance and dynamics of *Vanilla* populations and to provide guidelines for their preservation.

4. Vanilla biodiversity in cultivated conditions

Vanilla is the only orchid with a significant economic importance in food industry. It is cultivated for its aromatic fruit, a character restricted to some species from the American continent (Soto Arenas & Cameron, 2003). Only two species are grown to produce commercial vanilla: *V. planifolia* and *V. ×tahitensis*; with *V. planifolia* providing 95% of the world production, mainly originating from Madagascar, Indonesia, Comoros, Uganda and India (Roux-Cuvelier & Grisoni, 2010). Biodiversity in cultivated conditions depends on the level of diversity originally introduced and on cultivation practices used in different countries during domestication. Vanilla crops are established from stem cuttings of 8–12 nodes, collected from healthy and vigorous vines (Bory et al., 2008b; Bouriquet, 1954a; Purseglove et al., 1981; Soto Arenas & Cameron, 2003; Stehlé, 1952). As natural pollinators are absent in the areas of vanilla production, pollination is performed by hand following a simple method discovered by the slave Edmond Albius in Reunion Island in 1841 (Kahane et al., 2008). Given these cultivation practices, low levels of genetic diversity are expected in cultivation areas. However, for both species, different varieties, showing recognized but poorly defined morphological, agronomical and aromatic properties, are often cultivated by growers (Duval et al., 2006). Given the vegetative mode of propagation and the absence of pollinators, five hypotheses have been proposed to explain these variations (Bory et al., 2008b): (i) multiple introduction events, (ii) somatic mutations, (iii) sexual reproduction, (iv) polyploidy and (v) epigenetic modifications. In recent years, these hypotheses were explored, giving new insights on the processes involved during the dispersion and domestication of the two main cultivated *Vanilla* species. These results also give important clues to the understanding of *Vanilla* evolutionary processes in natural conditions.

4.1 *V. planifolia* in Reunion Island

The species *V. planifolia* originated in Mesoamerica (Portères, 1954). Some of the history of vanilla follows the history of chocolate because vanilla was gathered from the wild for use in flavoring chocolate beverages in the pre-Columbian Maya and Aztec cultures of southeastern Mexico and Central America. However, the Totonac people of Papantla in north-central Veracruz (Mexico) were probably the first group to cultivate *V. planifolia* (Lubinsky et al., 2011). The species *V. planifolia* has an interesting history of dispersal to other tropical regions between 27° N and 27° S latitudes (Lubinsky et al., 2008a). After the discovery of the Americas by C. Colombus, the whole history of *V. planifolia* dissemination, following the discoveries of manual pollination by the slave Edmond Albius in 1841 and curing process by E. Loupy and D. De Floris is intimately linked to Reunion Island (Kahane et al., 2008). From then, *V. planifolia* was renowned as 'Bourbon Vanilla' since it was produced originally from Reunion Island (from 1848) and later from a cartel of Indian Ocean Island producers (Madagascar, Reunion, Comoros and Seychelles).

The true origin of cultivated vanilla outside of Mexico was unclear until AFLP and microsatellite markers were used to elucidate the patterns of introduction of *V. planifolia.* These studies showed that most of the accessions cultivated today in the islands of the Indian Ocean and worldwide (Reunion Island, Madagascar, French Polynesia, French West

Indies, Mexico) and of different morphotypes (from Reunion 'Classique', 'Mexique', 'Sterile', 'Grosse Vanille' (table 3) and from Mexico 'Mansa', 'Acamaya', 'Mestiza') (Bory et al., 2008c; Lubinsky et al., 2008a) derive from a single introduced genotype. It could correspond to the lectotype that was introduced, early in the nineteenth century, by the Marquis of Blandford into the collection of Charles Greville at Paddington (UK) (Portères, 1954). Cuttings were sent to the botanical gardens of Paris (France) and Antwerp (Belgium) from where these specimens were disseminated to Reunion Island (by the ordinance officer of Bourbon, Marchant) and then worldwide (Bory et al., 2008b; Kahane et al., 2008).

Consequently, cultivated accessions in Reunion Island exhibit extremely low levels of genetic diversity and have evolved by the accumulation of point mutations through vegetative multiplication (Bory et al., 2008c) (table 3). Maximum genetic distance (Dmax) was 0.106 and the majority of the polymorphic AFLP bands revealed had frequencies in the extreme (0-10% and 90-100%) ranges, therefore corresponding to rare AFLP alleles (presence or absence) a pattern typical of point mutations (Bory et al., 2008c). One peculiar and rare phenotype 'Aiguille' found in Reunion Island was shown to result from sexual reproduction (selfing) (Bory et al., 2008c) (table 3) as its AFLP pattern fell within a group of selfed progeny with Dmax=0.140 and showed a strong pattern of segregation bands. The hypothesis was that it resulted from manual self-pollination and subsequent seed germination from a forgotten pod (Bory et al., 2008c). Flow cytometry, microdensitometry, chromosome counts and stomatal lengths showed that polyploidization has been actively involved in the diversification of *V. planifolia* in Reunion Island (Bory et al., 2008a). Three ploidy levels (2x, 3x, 4x) were revealed that allowed to explain the features of the 'Sterile' type which is auto-triploid and of the 'Grosse Vanille' type, auto-tetraploid (Bory et al., 2008a). It was suggested that these resulted from the production of non-reduced gametes during the course of manual self-pollination performed by growers (Bory et al., 2010; Bory et al., 2008a).

As the particular phenotype 'Mexique' encountered in Reunion could not be explained by genetic or cytogenetic variations, we tested whether it could have resulted from epigenetic modifications as some studies showed that morphological variations in clonal populations could be explained by a combination of genetic and epigenetic factors (Imazio et al., 2002). Epigenetics corresponds to reversible but heritable modifications of gene expression without changes in the nucleotidic sequence (Mathieu et al., 2007; Wu & Morris, 2001), such as DNA methylation (Finnegan et al., 1998). Epigenetic modifications are heritable (Akimoto et al., 2007; Finnegan et al., 1996; Grant-Downton & Dickinson, 2006; Martienssen & Colot, 2001) and transmitted as well as by asexual propagation (Peraza-Echevarria et al., 2001).

Sometimes, a phenotypic reversion correlated with demethylation of the epi-mutated gene can occur and its expression is restored (Jaligot et al., 2004). These epigenetic mutations have important phenotypic as well as evolutionary consequences, this representing a current field of investigation (Finnegan, 2001; Kalisz & Purugganan, 2004; B. Liu & Wendel, 2003). DNA methylation proceeds by the addition in a newly replicated DNA of a methyl group by a DNA methlytransferase (Finnegan et al., 1998; Martienssen & Colot, 2001). Cytosine is the most frequently methlylated base, resulting in 5-methylcytosine formation (^{5m}C) (Martienssen & Colot, 2001). Plant methylation is restricted to the nuclear genome and is concentrated in repeated sequence regions (Finnegan et al., 1998). Methylation is implied in many biological processes such as 'gene silencing', mobile DNA elements control, DNA replication duration, chromosome structure determination, and mutation frequency increase (Finnegan et al., 1998; Paszkowski & Whitham, 2001). Many spontaneous or induced epimutations are known in maize, Arabidopsis and other plant species and are responsible

Morphotypes	Characteristics	Diversity/genetics	Origin
'Classique'	The most cultivated type	Point mutations Dmax = 0.106	Mexico then Antwerp Botanical Gardens
'Aiguille'	Slender leaves and thin pods	As self progenies Dmax=0.140	Selfing of 'Classique'
'Sterile'	'Classique', but self-sterile	Same AFLP profile as 'Classique', auto-triploid (3x)	Selfing of 'Classique', unreduced gamete (2n x n)
'Grosse Vanille'	Bigger leaves, stems, flowers and fruits than 'Classique'	Same AFLP profile as 'Classique', auto-tetraploid (4x)	Selfing of 'Classique', unreduced gametes (2n x 2n)
'Mexique'	Darker bluish leaves with central gutter and curved sides, cylindrical pods	Same AFLP and MSAP profile as 'Classique'	Epigenetic or genetic single dominant mutation with pleiotropic effects

Table 3. *V. planifolia* morphotypes encountered in Reunion Island and their description.

Accession	Morphotype	Collection	Accession	Morphotype	Collection
CR0217	'Classique'	Provanille 3A11	CR0493	'Mexique'	Provanille 15A8
CR0218		Provanille 3A11	CR0494		Provanille 15A8
CR0219		Provanille 3A11	CR0495		Provanille 15A8
CR0343	'Classique'	Provanille 6A8	CR0334	'Mexique'	Provanille 6A5
CR0344		Provanille 6A8	CR0335		Provanille 6A5
CR0345		Provanille 6A8	CR0336		Provanille 6A5
CR0457	'Classique'	Provanille 15A6	CR0337	'Mexique'	Provanille 6A6
CR0458		Provanille 15A6	CR0338		Provanille 6A6
CR0459		Provanille 15A6	CR0339		Provanille 6A6
CR0563	'Classique'	Provanille 16B2	CR0001	'Mexique'	BRC Vatel
CR0564		Provanille 16B2	CR0002	'Mexique'	BRC Vatel
CR0565		Provanille 16B2	CR0627	'Mexique'	BRC Vatel StP
CR0340	'Classique'	Provanille 6A7	CR0649	'Mexique'	BRC Vatel StP
CR0341		Provanille 6A7	CR0632	'Mexique'	BRC Vatel StP
CR0342		Provanille 6A7	CR0711	'Classique'	BRC Vatel SteR
CR0647	'Classique'	BRC Vatel StP	CR0714	'Classique'	BRC Vatel SteR
CR0650	'Classique'	BRC Vatel StP			

Table 4. *V. planifolia* Reunion Island accessions surveyed in the MSAP analysis (StP: Saint Philippe; SteR: Ste Rose).

for the generation of mutant phenotypes (Finnegan et al., 1996; Martienssen & Colot, 2001). To assess whether 'Mexique' morphotypes might have resulted from epigenetic modifications, we selected the MSAP (Methylation-sensitive amplified polymorphism) method (Reyna-López et al., 1997), an AFLP-derived methodology which allows the visualization of a large number of markers revealing cytosine methylation state at each digestion site, without any *a priori* knowledge of genomic sequences. MSAP analyses were performed on a sample of 'Classique' and 'Mexique' accessions (table 4). Twenty-four accessions were collected in the collection of Provanille in Bras-Panon (Reunion Island), corresponding to 8 varieties with three cuttings. This was to verify if genetic or methylation polymorphism, if existing, is transmitted through vegetative multiplication. Others were

collected in vanilla plantations in Reunion Island (St-Philippe or Ste-Rose) and are maintained in the BRC Vatel collection.
We used the restriction enzyme *Eco*RI as well as *Msp*I and *Hpa*II, isochizomers that cut the same restriction site CCGG but show different sensitivity to methylation (table 5). The MSAP methodology used was as described in (Reyna-López et al., 1997). *Hpa*II digests were repeated twice. The adaptators used are presented in table 6 and 8 *Eco/Hpa* primer combinations were used for selective amplification.

	*Eco*RI/*Hpa*II	*Eco*RI/*Msp*I	CCGG methylation
Case number 1	1	1	CCGG
Case number 2	1	0	^{5hm}CCGG
Case number 3	0	1	C^{5m}CGG
Case number 4	0	0	^{5m}C^{5m}CGG or ^{5m}CCGG

Table 5. Methylation sensitivity of *Hpa*II and *Msp*I (^{m} : methylation; ^{hm} : hemimethylation).

The comparison of the profiles from the amplification after DNA digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I gives informations on the methylation status of the internal cytosine in sequence CCGG (table 5). For example a band present in the *Msp*I profile and absent in *Hpa*II indicates a methylation of the internal cytosine, whereas the opposite situation indicates an hemimethylation of the external cytosine. A methylation event was considered as polymorphic when at least one accession differed from the others in its profile.

Name	Sequence (5'-3')
Double strand adaptators	
Ad EcoRI1	CTC GTA GAC TGC GTA CC
Ad EcoRI2	AAT TGG TAC GCA GTC
Ad HpaII1	GAT CAT GAG TCC TGC T
Ad HpaII2	CGA GCA GGA CTC ATG A
Pre-amplification primers	
Eco-A	GAC TGC GTA CCA ATT CA
Hpa-A	TCA TGA GTC CTG CTC GGA
Selective amplification primers	
Eco-AC	GAC TGC GTA CCA ATT CAC
Eco-AG	GAC TGC GTA CCA ATT CAG
Hpa-ATT	ATC ATG AGT CCT GT CGG ATT
Hpa-ATG	ATC ATG AGT CCT GT CGG ATG
Hpa-AAC	ATC ATG AGT CCT GT CGG AAC
Hpa-AAG	ATC ATG AGT CCT GT CGG AAG

Table 6. Adaptator and primer sequences used in MSAP analysis.

Between 48 and 70 fragments were revealed by primer combination. On the 483 CCGG sites observed, 188 were non methylated (38.9%), 36 were methylated (7.45%), with 5 sites only presenting methylation polymorphisms (1.03%) in 4 accessions. Accessions CR0340 and CR0341 were hypomethylated, they showed bands in both their *Hpa*II and *Msp*I profiles whereas the other accessions only presented these bands with *Msp*I. CR0340 was

hypomethylated at locus Eco-AG/Hpa-AAC/98bp and CR0341 at locus Eco-AC/Hpa-ATT/426bp. Accessions CR0632 and CR0711 were hypermethylated, they presented some bands in their *Msp*I profiles whereas the other accessions presented these bands in both their *Hpa*II and *Msp*I profiles. Accession CR0632 was hypermethylated at locus *Eco*-AG/*Hpa*-ATG/205bp and at locus *Eco*-AG/*Hpa*-AAC/382bp. Finally, accession CR0711 was hypermethylated at locus *Eco*-AG/*Hpa*-AAG/393bp.

These results showed that methylation is present in *V. planifolia* genome, with 7.45% of the fragments revealed being methylated. This value is in accordance with methylation rates reported in banana (7,5%, (Noyer et al., 2005)), but less than what is revealed in other conventionally propagated plant species such as rice (16.3%, (Xiong et al., 1999)), other bananas (18.4%, (Peraza-Echeverria et al., 2001)), apple (25%, (Xu et al., 2000)) and cotton (32%, (Keyte et al., 2006)).

A limited amount of methylation polymorphism (1%) was detected among 'Classique' and 'Mexique' accessions but the methylation patterns revealed were accession specific. Even for CR0340/0341/0342 which are three clones of the same accession, two different methylation polymorphisms were revealed in CR0340 and CR0341 and none in CR0342, showing that these methylation patterns are either not transmitted trough asexual propagation, or have appeared after clonal propagation. In all cases, no methylation marker was identified which could allow to specifically distinguish the 'Classique' and 'Mexique' morphotypes. A similar conclusion was obtained in studies performed on vegetatively propagated plants such as banana (Baurens et al., 2003; Noyer et al., 2005). Methylation polymorphisms were revealed but could not be correlated to morphological variations.

We therefore conclude that the 'Mexique' morphotype showing no detectable AFLP or MSAP polymorphism is most probably the result of a limited genetic or epigenetic dominant mutation event with pleiotropic effects.

4.2 *V. ×tahitensis* in French Polynesia

The mysterious history of the origin of *V. ×tahitensis*, the so called Tahitian vanilla, has partly been solved. As opposed to its allied species (*V. planifolia*) it cannot be found wild in tropical American forests (Moore, 1993; Portères, 1954; Soto Arenas & Cameron, 2003) but was described from cultivated material found in the Island of Raiatea (Lubinsky et al., 2008b), where it had been introduced via the botanical garden of Papeete from the Philipines in 1848 (Soto Arenas & Dressler, 2010). Molecular sequencing (ITS and cpDNA) have recently shown that *V. ×tahitensis* would be a hybrid, intentional or not between *V. planifolia* and *V. odorata* dating from vanilla exploitation by Mayas in Mesoamerica between years 1359-1500 (Lubinsky et al., 2008b).

As much as 18 different morphotypes are described in *V. ×tahitensis* in French Polynesia beside the most widely cultivated type 'Tahiti' (Lepers-Andrzejewski et al., 2011a). These include: 'Haapape' (the second most cultivated type because of its bigger fruits), 'Tahiti Court', 'Tiarei', 'Ofe Ofe', 'Oviri', 'Parahurahu' and 'Sterile'. A study of 16 different accessions using AFLP markers revealed a Dmax value of 0.150, a slightly higher value than what was revealed in *V. planifolia*. All accessions had patterns related to that of 'Tahiti' (either identical or showing missing bands) which led the authors to conclude of a single introduction event in French Polynesia of a 'Tahiti' vine, consistently with the fact that this accession is the oldest one recorded in Polynesia (Lepers-Andrzejewski et al., 2011a). Ten accessions showed more or less the AFLP profile of 'Tahiti'. These included 'Haapape' and 'Tiarei', which were shown to be autotetraploids based on flow cytometry and chromosome

counts (Lepers-Andrzejewski et al., 2011b). Similarly as in *V. planifolia* (Bory et al., 2008a), 'Sterile' morphotypes in *V.* ×*tahitensis* were also related to autotriploidy (Lepers-Andrzejewski et al., 2011b). It was hypothesized that they originated from a cross between the two most cultivated morphotypes 'Tahiti' (2x) and 'Haapape' (4x) (Lepers-Andrzejewski et al., 2011b). The remaining accessions showed a pattern related to 'Tahiti' but with 15 to 30 missing bands (Lepers-Andrzejewski et al., 2011a), a pattern consistent with segregation, as shown in *V. planifolia* for the 'Aiguille' morphotype or selfed progenies (Bory et al., 2010). For these accessions, graphical genotypes were constructed based an AFLP 'Tahiti' map and showed that morphotypes such as 'Parahurahu', 'Rearea', 'Oviri' and 'Tahiti court' displayed patterns consistent with an origin via self-pollination of 'Tahiti' (one single recombination event per bivalent) whereas others such as 'Popoti' and 'Paraauti' most probably resulted from a second generation of self-pollination (two recombinations events in the same bivalent) (Lepers-Andrzejewski et al., 2011a).

4.3 Conclusions

These results therefore highlight two different domestication models. In both cases, the genetic base of the cultivated material is very narrow with obviously a single genotype introduced ('Classique' *V. planifolia* in Reunion Island and other cultivation areas; 'Tahiti' *V.* ×*tahitensis* in French Polynesia). Genetic variation revealed is however slightly higher in *V.* ×*tahitensis* than in *V. planifolia* because most of *V.* ×*tahitensis* morphotypes have resulted from selfing of the original 'Tahiti' (with sometimes more than one generation involved) (Lepers-Andrzejewski et al., 2011a). Only one rare case of self-pollination ('Aiguille') was detected in Reunion (Bory et al., 2010). This shows that deliberate or inadvertent seed germination has been strongly involved in the domestication of *V.* ×*tahitensis* in French Polynesia. In Reunion Island, the limited amount of variation revealed is more related to vegetative propagation and the consecutive accumulation of point mutations.

In both cases however, a noticeable diversification was achieved through polyploidy. Auto-tetraploidy generated varieties with bigger leaves and fruits, and autotriploidy generated self-sterile individuals. It is noteworthy that self-sterile *V. planifolia* varieties were also described in Mexico ('Oreja de Burro') (Castillo Martinez & Engleman, 1993; Soto Arenas & Dressler, 2010). It is most likely that these have resulted as well from autotriploidy. These results, as well as those that surveyed genome sizes in a wide range of *Vanilla* species (Bory et al., 2010) provide converging evidences for the importance of polyploidy and genome rearrangements during *Vanilla* evolution. Polyploidy can be of major importance in cultivation as well as in natural populations as triploidy and to a certain extent tetraploidy can be responsible for dramatic loss in fruit set. Further work is therefore needed to assess polyploidization consequences on *Vanilla* reproductive biology.

5. *Vanilla* genome dynamics

Concordant data obtained on *V. planifolia* as well as *V.* ×*tahitensis* demonstrated an abnormal mitotic behaviour in the *Vanilla* genus, with a combination of somatic aneuploidy and partial endoreplication (Bory et al., 2008a; Lepers-Andrzejewski et al., 2011b).

5.1 Somatic aneuploidy

Most data in the literature give a basic number n=16 for *V. planifolia* with 2n= 32 (Chardard, 1963; Heim, 1954; Hoffmann, 1929, 1930; Martin, 1963). Hurel-Py (1938) was the first to show

the existence of a variable number of chromosomes in differentiated cells (13 to 32 chromosomes). Similarly, Nair & Ravindran (1994) described an important variation in chromosome numbers, from 20 to 32 with 28 being the most encountered. Recent analyses confirmed the existence of such somatic hypo-aneuploidy (i.e. chromosome number is always below an exact multiple of the usually haploid number) in root tip cells of *V. planifolia* (Bory et al., 2008a), *V.* ×*tahitensis* (Lepers-Andrzejewski et al., 2011b) as well as other *Vanilla* species (Bory, 2007). This aneuploidy could be explained by somatic associations of chromosomes (Nair & Ravindran, 1994) but as well by chromatin elimination (Lepers-Andrzejewski et al., 2011b). Interestingly, it was recently demonstrated that somatic aneuploidy is regulated between somatic and gametic cells in *V.* ×*tahitensis*, with the full genome complement present in germ cells (Lepers-Andrzejewski et al., 2011b). This suggests that a regulatory mechanism functions during meiosis to stabilize the genome and chromosome number

5.2 Progressively partial endoreplication

Flow cytometry genome size estimates and chromosome counts have been successfully used to demonstrate the occurrence of diploid, triploid and tetraploid accessions of *V. planifolia* in Reunion Island (Bory et al., 2008a) and *V.* ×*tahitensis* in French Polynesia (Lepers-Andrzejewski et al., 2011b). Genome size variations were also demonstrated in some other species of the *Vanilla* genus (Bory et al., 2010). Flow cytometry revealed endoreplication in somatic cells of *V. planifolia* and *V.* ×*tahitensis*. In *V. planifolia* the marginal replication ratio, which is the ratio between each peak position, was irregular with 1.43, 1.63, 1.76, 1.82 instead of 2.00 (Bory et al., 2008a). In *V.* ×*tahitensis* it was 1.38, 1.65, 1.77, 1.79 and 1.81 (Lepers-Andrzejewski et al., 2011b). The almost perfect linearity found between DNA content and the number of endoreplication cycles suggested that the same genome part (or chromosome batch) (P, figure 3) is amplified at each cycle. A matrix of only 43.73% and 38% of the holoploid nucleus is replicated at each cycle in *V. planifolia* and *V.* ×*tahitensis*, respectively (Bory et al., 2008a; Lepers-Andrzejewski et al., 2011b).

More importantly, this phenomenon is apparently present in all the *Vanilla* species surveyed so far. Flow cytometry genome size estimates for 38 accessions representing 17 different *Vanilla* species and 3 artificial inter-specific hybrids revealed, for each accession, fluorescence histograms with five endoreplicated peaks and the marginal replication ratio was still irregular (from 1.5 to 1.8 instead of 2) (Bory et al., 2010). Nothing is known concerning the mechanisms in play, whether it results from partial replication of the DNA or excision of DNA (possibly chromatin elimination) following whole genome replication, but they occur in many orchids (Bory et al., 2008a). It will be important in the near future to gain knowledge on this developmentally regulated ''progressively partial endoreplication'' phenomenon unique to orchids. Available data already show that it is vegetatively, as well as sexually transmitted as demonstrated by surveying interspecific hybrids, such as the natural hybrid *V.* ×*tahitensis* (Lepers-Andrzejewski et al., 2011b) and artificial hybrids (*V. planifolia* × *V. planifolia*, *V. planifolia* × *V.* ×*tahitensis*, *V. planifolia* × *V. phaeantha)* (Bory et al., 2010). This phenomenon is technically important as the first peak (2C) is often very small, and this was shown to be responsible for considerable errors in the genome size estimates that have been published in the literature for *Vanilla* species (Bory et al., 2008a; Lepers-Andrzejewski et al., 2011b). This phenomenon is also evolutionary important as it was shown to be a source of polyploidization in many plant species. However it cannot itself explain the origin of autotetraploid types in *V. planifolia* and *V.* ×*tahitensis* as these have

exactly double the amount of DNA than their diploids counterparts, unless endoreplication in meristematic cells is regulated (Lepers-Andrzejewski et al., 2011b).

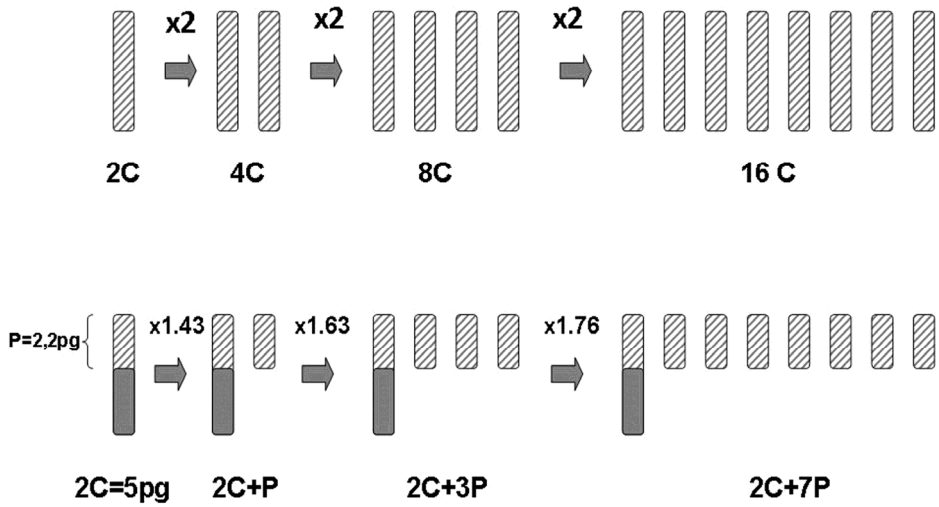

Fig. 3. Partial progressive endoreplication in *V. planifolia* (below) as compared to normal endoreplication (above). The replicated part (P) of the *V. planifolia* genome is indicated (hatched).

6. Conclusion

Although considerable progress has been made in recent years in the precision of the taxonomy and the discovery of evolutionary processes in the *Vanilla* genus (reproduction, genetic diversity, polyploidy, hybridization), many questions remain unanswered. These include elucidating the complex processes involved in genome dynamics and its possible implications on the genus diversification. Evolutionary pathways of important traits in the genus such as self-pollination ability and aromatic compounds accumulation in fruits, which are major targets for vanilla breeding, will need to be surveyed. Self-pollination appears as an ancestral character in the genus, shared by species from group α and early diverging species from group β. Furthermore, although allied genera possess aromatic fruit, this character is found in *Vanilla* within American group β, but not in ancestral American nor in more recent species from Africa and Asia. The aromatic character of both flowers and fruit in *Vanilla* has evolved in a specialized relationship with euglossine bees involved in both flower pollination and fruit dispersion. This represents an exciting further area of investigation. Molecular and cytogenetic studies will have to be combined with morphological, history traits and ecological assessments to provide a thorough revision of the genus taxonomy. In particular, more data is needed to fully characterize the reproductive biology of *Vanilla* species and its implication on the levels of genetic diversity in natural populations. This will be essential to provide conservation guidelines for the many endangered species of the genus.

7. References

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1.3 Présentation des vanilliers aphylles

Comme souligné précédemment dans la revue bibliographique qui précède, des données sont nécessaires pour caractériser la biologie de la reproduction des vanilliers et ses conséquences sur les niveaux de diversité génétique, en particulier les espèces aphylles *V. humblotii* et *V. roscheri* du sud-ouest de l'océan Indien.

En dehors de quelques travaux réalisés sur certaines espèces américaines (Soto Arenas 1999, 2010; Soto Arenas and Cameron 2003; Soto Arenas and Cribb 2010; Soto Arenas and Dressler 2010; Householder et al. 2010; Nielsen 2000; Nielsen and Siegismund 1999; Lubinsky et al. 2008; Lubinsky et al. 2006), la plupart des espèces sauvages de vanilliers restent très mal connues, tant d'un point de vue de la biologie de la reproduction que de leur diversité génétique. Les écosystèmes tropicaux sont menacés (Myers 1988; Myers 1992; Myers 1994; Myers et al. 2000; Laurance 1999; Laurance et al. 2000; Antonelli and Sanmartín 2011), en particulier les forêts tropicales sèches (Janzen 1988; Miles et al. 2006; Hoekstra et al. 2005) qui abritent les vanilliers aphylles (correspondant à la section Aphyllae du genre *Vanilla* établie par Rolfe (1896)). Les espèces aphylles doivent donc potentiellement être considérées comme des espèces menacées dans le genre *Vanilla* et nécessitent d'être caractérisées à la fois génétiquement et écologiquement afin de pouvoir suggérer des mesures de conservation appropriées.

La Section Aphyllae du genre *Vanilla*

Le groupe des vanilliers aphylles comprend au total 18 espèces dépourvues de feuilles ou possédant des feuilles vestigiales réduites à des écailles (Rolfe 1896; Portères 1954b). Leur distribution est à l'image de celle des foliées présentes dans les zones intertropicales des trois continents américain, africain et asiatique (Portères 1954b) (Figure 1).

Possédant des surfaces foliaires réduites diminuant l'évapotranspiration, les vanilliers aphylles sont particulièrement bien adaptés aux conditions de sécheresse de basse à moyenne altitude (jusqu'à

600m). Ils croissent principalement sur des buissons littoraux, dans des forêts tropophiles très sèches, sur des sols sableux et sur des rochers mais se rencontrent également au sein de formations hydro-halophytiques pour certaines espèces américaines (Portères 1954a), aussi en forêt tropicale humide (*V. madagascariensis* Rolfe à Madagascar) comme la majorité des espèces du genre *Vanilla,* ou mésophile (*V. humblotii* à Mayotte (archipel des Comores)), ce qui suggère une plasticité écologique élevée de ces espèces sauvages.

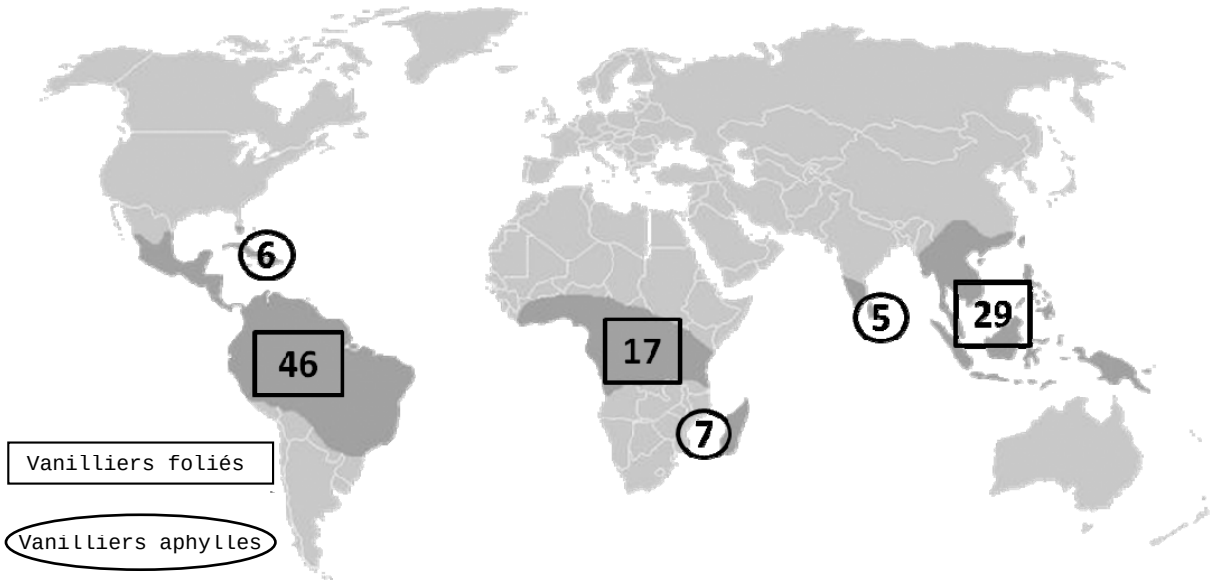

Figure 4 Distribution mondiale des 110 espèces du genre *Vanilla*, d'après Portères (1954). Le nombre d'espèces foliées et le nombre d'espèces aphylles est précisé pour chacun des trois continents (américain, africain et asiatique).

De manière remarquable, les vanilliers aphylles de chacun des trois groupes continentaux ont une distribution insulaire, avec dans chaque groupe, des espèces présentes sur les littoraux continentaux en disjonction d'une répartition insulaire: *V. roscheri* (une des deux espèces de notre étude) sur le littoral sud-est africain est présente aux îles Pemba et Zanzibar, *V. parishii* Rchb. f. à la fois présente en Inde et au Sri Lanka et *V. barbellata* Rchb. f. décrite en Floride est aussi présente dans les îles de Cuba, Haïti et Puerto Rico (Portères 1954b). De récentes analyses phylogéographiques couplées à une datation moléculaire ont montré le caractère polyphylétique de la section Aphyllae (Figure 1) (Bouetard et al. 2010). L'aphyllie serait apparue indépendamment au moins trois fois dans l'évolution

du genre à partir d'ancêtres foliés du Vieux Continent au cours du Miocène (Bouetard et al. 2010). Ces analyses ont montré l'importance des phénomènes de migration à longue distance (transocéanique), notamment celui nécessaire à l'apparition des aphylles américaines à partir d'ancêtre africains, à une époque où les masses continentales étaient déjà bien individualisées. Les études réalisées sur les vanilliers aphylles américains *V. barbellata* et *V. claviculata* (W. Wright) Sw. à Puerto Rico, suggèrent effectivement une diversification probablement récente de ces espèces, avec l'existence d'hybrides interspécifiques là où ces espèces se trouvent en sympatrie (Nielsen and Siegismund 1999; Nielsen 2000). Au regard de la proximité géographique de groupes d'espèces aphylles et foliées et de l'originalité de la distribution des espèces aphylles, Portères (1954) avait déjà caractérisé l'aphyllie comme un trait de convergence adaptative aux conditions de sécheresse et suggéré une origine polyphylétique des espèces aphylles.

Par ailleurs, l'analyse de la diversité morphologique de certaines accessions de l'espèce foliée américaine *V. bahiana*, disponibles au CRB Vatel, montre l'existence d'accessions présentant des feuilles de taille réduite (Figure 5 C-D), et récemment, des populations ont été observées en zone littorale sèche du nord-est brésilien, en bordure de plage (Vaucher, com. pers.), dans un habitat similaire aux vanilliers aphylles connus, comme si une évolution vers l'aphyllie était en marche chez certaines populations de cette espèce. De plus, Soto Arenas and Cameron (2003) rapportent la singularité de la réduction des feuilles de l'espèce *V. penicillata* Garay & Dunst distribuée en Amérique Du Sud, mais cette espèce est non apparentée aux autres espèces aphylles notamment américaines dont l'origine est afro-asiatique. Ces remarques suggèrent que l'apparition du caractère aphylle est probablement un phénomène récurent à l'échelle du genre et donc que les distinctions entre espèces aphylle et foliée restent difficiles à interpréter, et vraisemblablement encore à élucider (Figure 5).

Figure 5 Variabilité morphologique des appareils végétatifs de quelques accessions du CRB Vatel. Deux espèces à grandes feuilles : A, l'espèce cultivée *V. planifolia* (CR0045) et B, *V. pompona* (CR0033). C-D, respectivement les accessions CR0668 et CR0087 de l'espèce *V. bahiana,* présentant un polymorphisme de la taille des feuilles. Deux exemples de vanilliers aphylles : E, *V. humblotii* (CR0108) et F, *V. cf dilloniana* (CR0081).

Les vanilliers aphylles de l'océan Indien

En Afrique, on dénombre 17 espèces de vanilliers foliés et sept aphylles (Portères 1954b) (Figure 4 et 6). A l'échelle de l'océan Indien, un pattern de différentiation morphologique se dessine au sein des

sept espèces aphylles avec l'existence de deux groupes différentiés par deux morphes floraux, qui pourraient potentiellement être associés à deux syndromes de pollinisation distincts (Figure 7) :

-les vanilliers aphylles aux fleurs jaunes (*V. humblotii* et *V. perrieri* Schltr.) (Figure 7 A-D) ;

-les vanilliers aphylles aux fleurs blanches (*V. roscheri*, *V. madagascariensis*, *V. decaryana* H. Perrier, *V. montagnacii* Portères et *V. phalaenopsis* Reichb. f. ex Van Houtte) (Figure 7 E-N). On notera l'absence de *V. montagnacii,* sur la planche ci-dessous (Figure 7). L'espèce est décrite à fleur blanche par Portères, mais à notre connaissance aucune illustration n'est disponible. En l'absence de description latine de *V. montagnacii*, Soto Arenas and Cribb (2010) ont rejeté l'existence de cette espèce, mais au regard de la diversité morphologique des espèces à fleur blanche, il n'est pas exclu qu'elle fasse partie d'une des accessions non identifiée du CRB Vatel (CR0699, CR694 ou CR0821 (Figure 7 L-N)) dont les morphologies sont différentes des deux autres espèces à fleur blanche de Madagascar, *V. madagascariensis* et *V. decaryana* identifiées par Cribb and Hermans (2009) et Lecoufle and Bosser (2011).

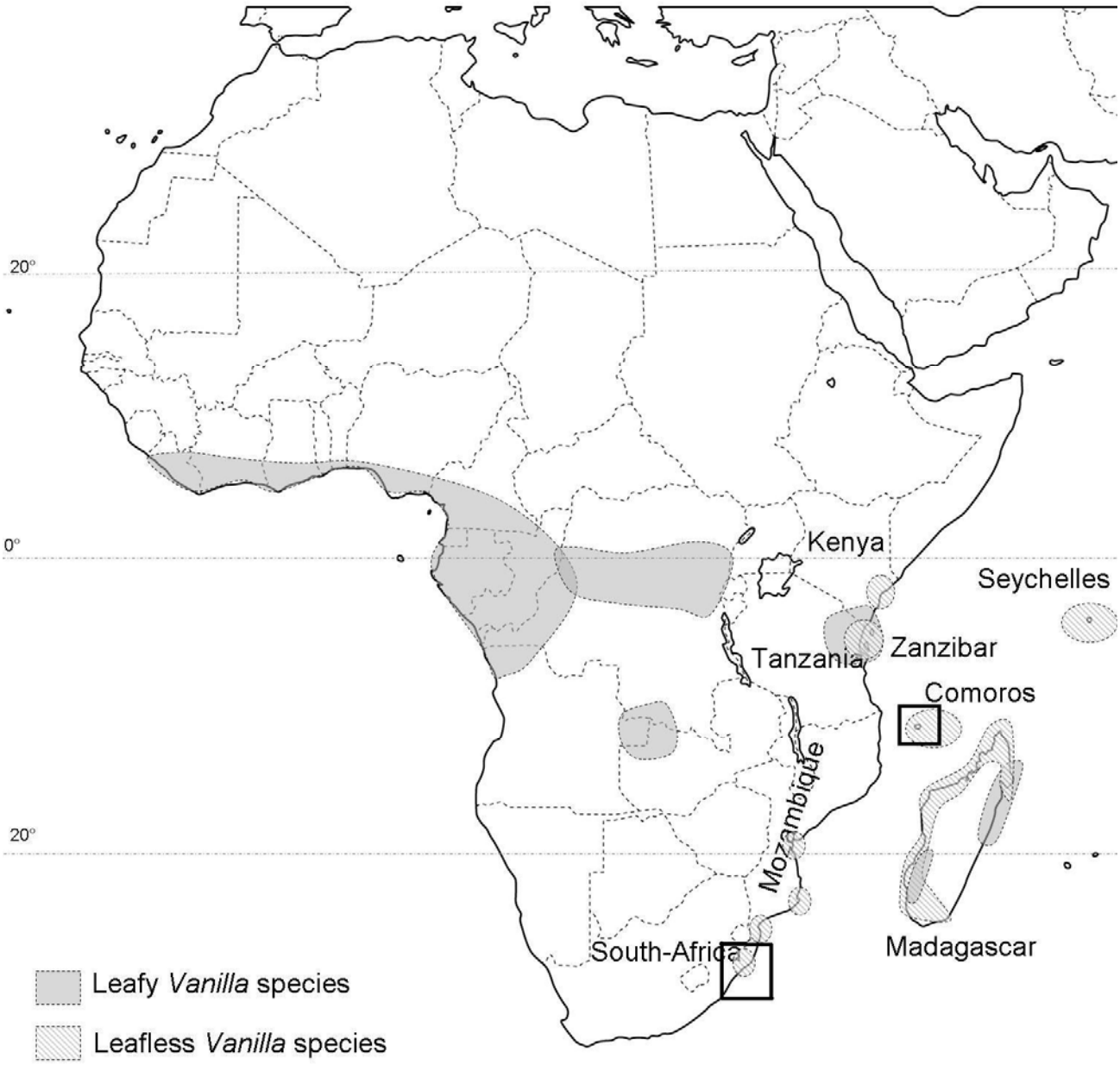

Figure 6 Distribution des vanilliers africains, avec en encadré les deux sites d'études des vanilliers aphylles de l'océan Indien, en Afrique du Sud et dans l'archipel des Comores (Krupko et al. 1954; Ward 1984; Beentje 1990; La Croix and Cribb 1995; Da Silva et al. 2004; Portères 1954b).

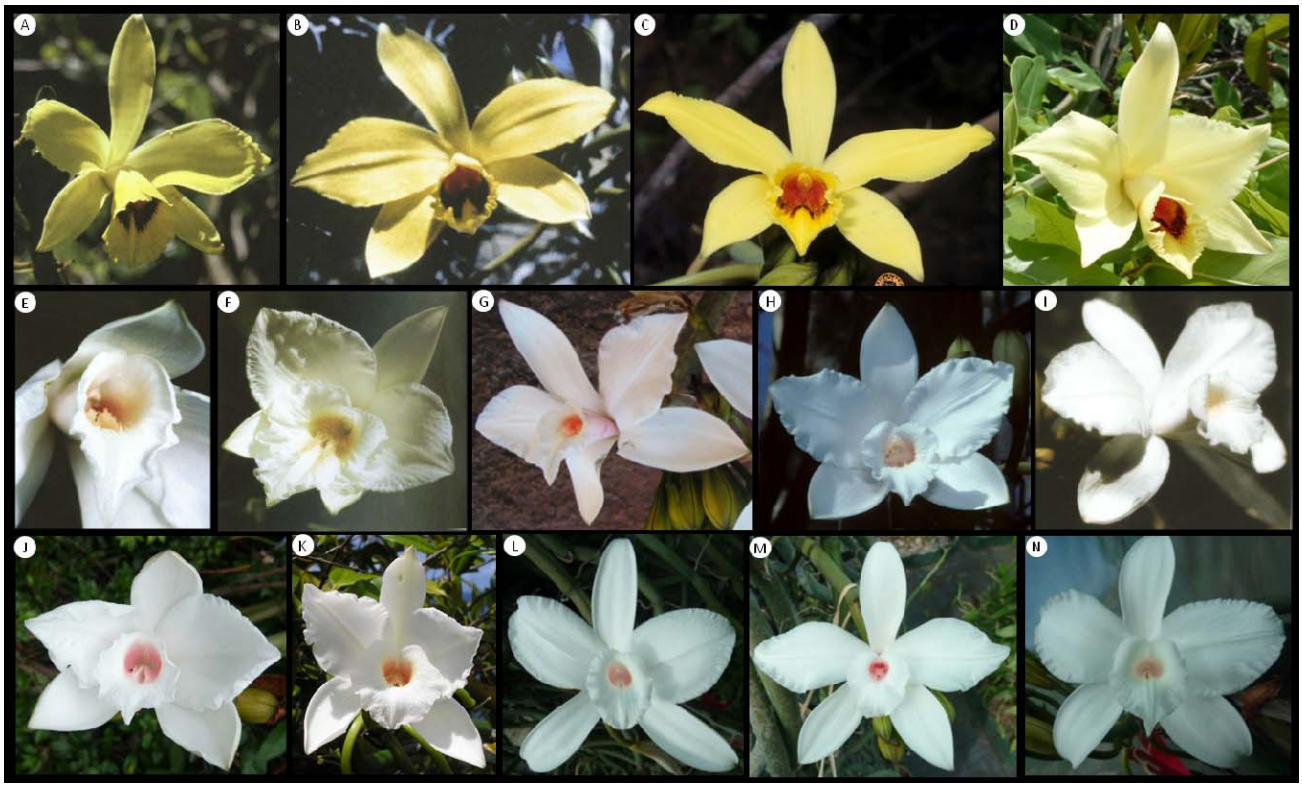

Figure 7 Diversité morphologique des fleurs de vanilliers aphylles de l'océan Indien. A-B, *Vanilla perrieri* à Madagascar. C-D, *Vanilla humblotii* d'origine non indiquée et à Mayotte. E-G, *Vanilla madagascariensis* et H-I, *Vanilla decaryana* à Madagascar. J, *Vanilla roscheri* en Afrique Du Sud. K, *Vanilla phalaenopsis* aux Seychelles L-N, *Vanilla sp.* (CR0699 d'origine non indiquée CR0694 probablement de Madagascar et CR0821 de Madagascar). (Photos A : Cribb et Hermans (2009) ; B, E, F et I : Lecoufle et Bosser (2011) ; C : Tarnaud (MNHN); D, J, L, M, N : Gigant (UMR PVBMT); G : Guitou (MNHN) ; H : Du Puy (MNHN); K : Pailler (UMR PVBMT)).

Les vanilliers aphylles de l'océan Indien forment un clade monophylétique récent dans l'évolution du genre, qui serait apparu il y a 4 à 15 millions d'années (Figure 1) à partir d'une migration à longue distance d'un ancêtre folié africain (Bouetard et al. 2010). Les analyses génétiques complémentaires intégrant un plus grand nombre d'accessions de la zone (Figure 8) ont cependant échoué à révéler un pattern de différentiation interspécifique (Gigant 2008). L'incapacité des analyses phylogénétiques à identifier un pattern de différentiation interspécifique reflète probablement l'origine récente de la diversification des vanilliers aphylles de l'océan Indien. Par ailleurs, Cameron (2011) a suggéré récemment que les espèces *V. roscheri, V. phalaenopsis* et *V. madagascariensis* aux fleurs blanches

pourraient être des variations morphologiques d'une seule et même espèce. Néanmoins, nos récentes comparaisons des accessions aphylles de l'océan Indien aux fleurs blanches disponibles au sein du CRB Vatel (dont certaines non-encore identifiées taxonomiquement), suggèrent la présence de plusieurs morphologies florales probablement suffisamment distinctes (Figure 7 : taille et forme du labelle, position et forme des ornementations du labelle, notamment de la zone pubescente) pour justifier l'existence de différents groupes taxonomiques au sein des espèces à fleur blanche de l'océan Indien.

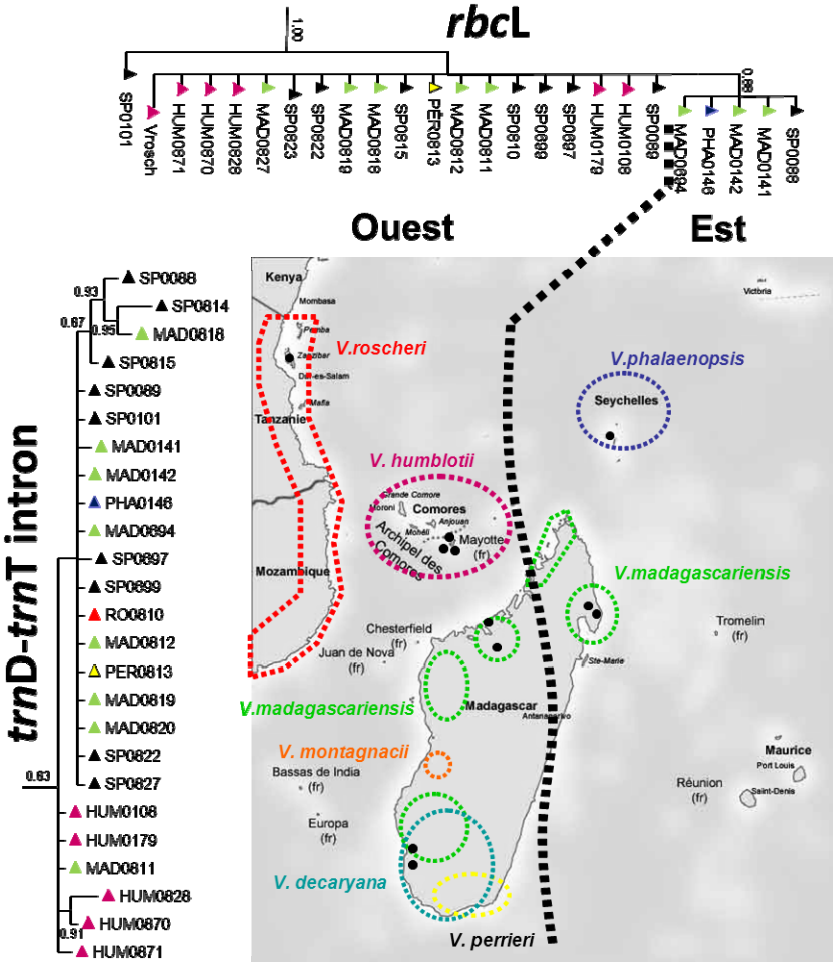

Figure 8 Pattern de différentiation phylogénétique des vanilliers aphylles de l'océan Indien couplé à leur distribution géographique d'après Gigant (2008). Les marqueurs chloroplastiques *trn*D-*trn*T (intergène) et *rbc*L (exon) ont été utilisés et suggèrent respectivement une différenciation des accessions de *V. humblotii*, ainsi qu'une différentiation Est/Ouest des accessions de la zone océan Indien sans relation avec leur origine taxonomique. Les indications sont données pour les espèces *V. madagascariensis* (MAD), *V. phalaenopsis* (PHA), *V. perrieri* (PER), *V. humblotii* (HUM) et *V. roscheri* (RO, Vrosch) mais l'identification reste incertaine pour de nombreuses accessions (SP).

Quoiqu'il en soit, aucune ambiguïté ne subsiste quant aux identifications taxonomiques de *V. roscheri* et *V. humblotii,* objets d'étude de cette thèse, puisqu'au sein des régions échantillonnées (Figure 4 et 6, Afrique Du Sud et Mayotte), une seule espèce est présente. Chacune de ces deux espèces est par ailleurs représentative d'un groupe de couleur florale.

***Vanilla humblotii,* espèce aphylle à fleur jaune endémique de l'archipel des Comores**

V. humblotii, telle que décrite par Portères (1954b), est une espèce aphylle dont la tige est épaisse, d'un vert glauque, ramifiée et verruqueuse, dont les entrenœuds mesurent 6 à 10 cm de long. Les écailles de 1-5 cm sont visibles au niveau des jeunes pousses, mais sont caduques disparaissent rapidement à l'apparition de nouveaux entrenœuds. Les inflorescences mesurent 25 à plus de 30 cm de long et portent 40 à 50 grandes fleurs d'un jaune-canari au labelle ondulé-frangé à l'avant et d'un rouge velouté et pubescent sur deux larges lignes à l'intérieur (Portères 1954b). A l'avant du disque mais pas sur les marges, peuvent être distinguées des lignes brunes rayonnantes arrondies. Le fruit cylindrique ou aplati, est bi-sillonné et mesure jusqu'à 25 cm de longueur (Portères 1954b).

V. humblotii se distribue dans l'archipel des Comores où des observations ou des collectes ont été réalisées en Grande Comore, à Anjouan et à Mayotte (Portères 1954b; Barthelat et al. 2006). Elle a été retrouvée croissant sur des milieux rocailleux parfois très exposés au soleil (Portères 1954b). A Anjouan, de récentes prospections ont suggéré que l'espèce était probablement en déclin voire en danger d'extinction dans son habitat naturel (L. Gigord, com. pers.). Par ailleurs, la végétation naturelle de l'ensemble de l'archipel des Comores est menacée par la disparition des habitats primaires. L'île la plus ancienne de l'archipel, Mayotte (374 km²), détient les reliques de forêts primaires les mieux préservées. La majorité de la diversité floristique de l'archipel est concentrée sur moins de 5% du territoire mahorais, retrouvée sur certaines pentes abruptes ou sur les crêtes d'anciens cônes volcaniques (Figure 9) (Vos 2004; Pascal et al. 2001). Les menaces principales qui pèsent sur la flore comorienne sont liées à la destruction de l'habitat forestier pour l'installation des

cultures et à l'exploitation non durable des forêts, aux invasions biologiques et parfois aux usages traditionnels (DAF 2002; Guéneau 2006). Par ailleurs, l'explosion démographique et le développement économique des îles accentuent d'autant plus la pression sur les espaces naturels (Trouillard et al. 2009).

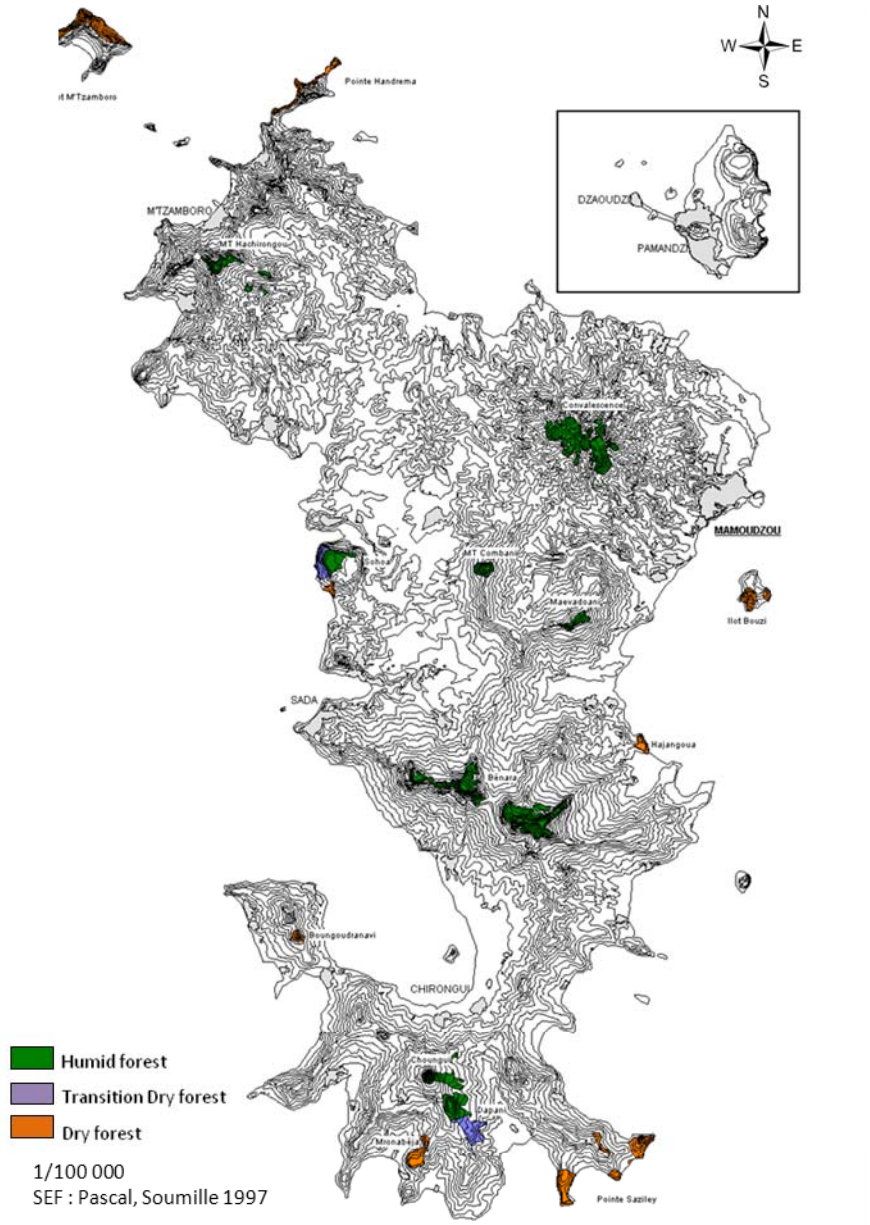

Figure 9 Distribution des reliquats forestiers à Mayotte en 1997 (DAF 2002; Pascal et al. 2001).

A Mayotte, *V. humblotii* est une espèce patrimoniale protégée par un arrêté préfectoral (AP n° 42/DAF/2006 3 mai 2006). La floraison et la fructification de *V. humblotii* ont été observées

récemment (Barthelat et al. 2006) (Figure 10), mais aucune donnée quantitative ni qualitative n'est disponible sur la reproduction naturelle, ni la diversité génétique de l'espèce à l'échelle de Mayotte, alors qu'il s'agit d'éléments essentiels pour la conservation de l'espèce.

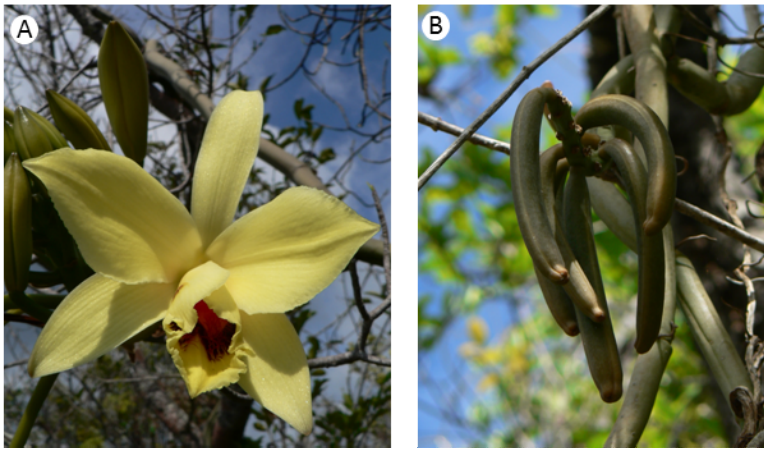

Figure 10 Clichés récents témoignant de l'existence de la reproduction naturelle de *V. humblotii* avec une fleur de *V. humblotii* (A) et la présence de fruits observée (B) (Photo: G. Viscardi CBNM).

En effet, plusieurs menaces persistent sur les populations naturelles de *V. humblotii* à Mayotte (Figure 11). Les populations se situent parfois sur des terrains privés et se confondent aux zones cultivées, essentiellement de manioc (*Manihot esculenta* Crantz, Euphorbiaceae), banane (*Musa sp.* L. Musaceae) et ambrevade (*Cajanus cajan* (L.) Millsp., Fabaceae) (Figure 11A et 11B). Les invasions biologiques à Mayotte font également partie du cortège de menaces qui pèsent sur les habitats naturels et/ou directement ou indirectement sur la survie des populations de *V. humblotii.* L'une des plus inquiétantes concerne l'espèce introduite *Lantana camara* L. (Verbenaceae). Cette peste végétale envahit les zones sèches et forme de denses buissons lianescents où aucune autre espèce n'arrive à prospérer (Pascal et al. 2001). L'exploitation illégale de l'igname endémique des Comores, *Dioscorea comorensis* (Figure 7C), très prisé par les populations locales, entraine une déforestation importante des zones sèches, qui laisse les terrains naturels jonchés de fosses étendues et profondes (Figure 11D) qui fragilisent les sols et facilitent la colonisation des pestes végétales, plus compétitives que les espèces indigènes pour la conquête du milieu laissé vacant.

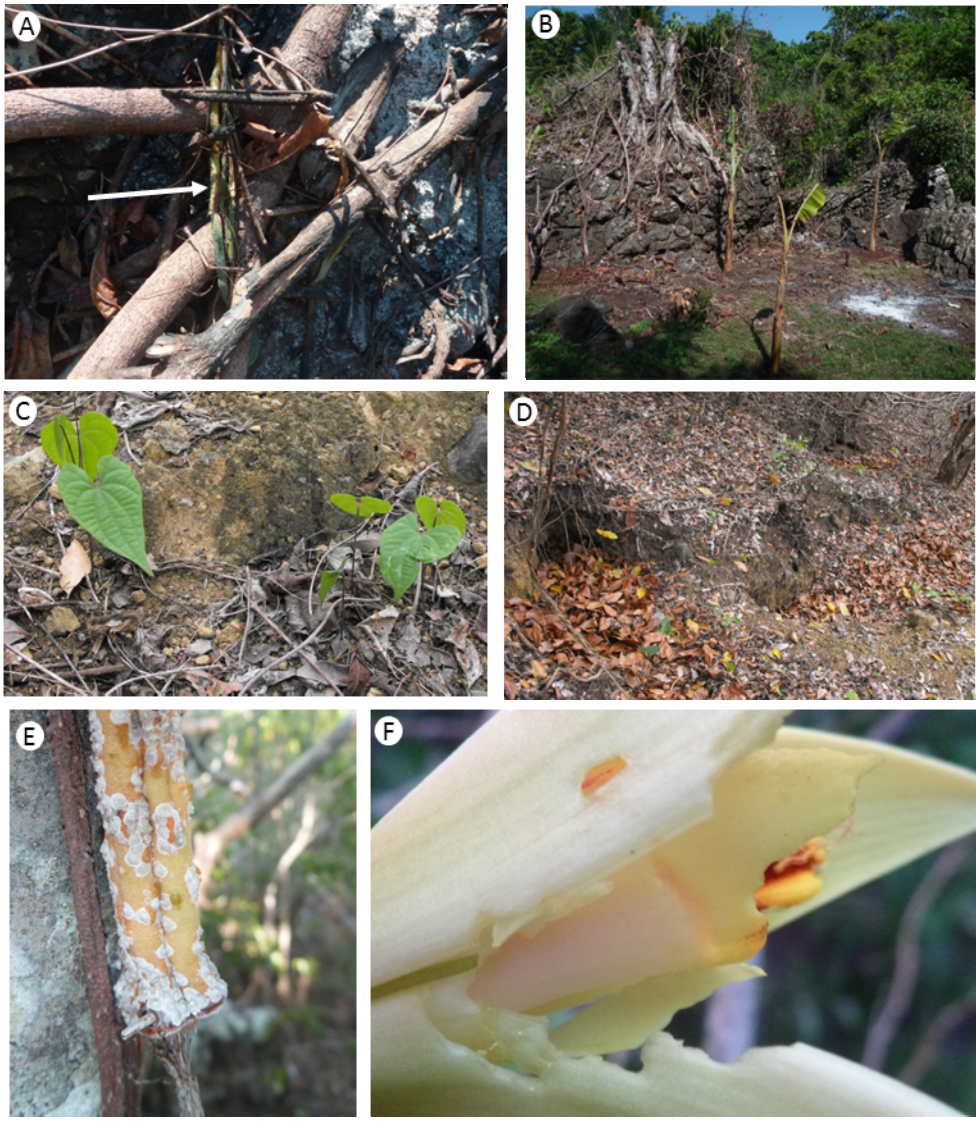

Figure 11 Menaces identifiées sur les populations de *V. humblotii*. (A) Fragment de liane de *V. humblotii* persistant parmi les débris de végétation abattus (Site Boungoudranavi). (B) installation d'une culture de bananier en saison des pluies, en lieu et place d'une ancienne population de *V. humblotii* (Site Boungoudranavi). (C) *Dioscorea comorensis* R. Knuth (Dioscoreaceae), igname endémique des Comores braconné. (D) Fosses laissées par les braconniers après le déterrement de tubercules de *Dioscorea comorensis*. (E) Symptomes de décoloration aigüe de la liane de *V. humblotii* après infestation de la cochenille *Conchaspis angraeci* Cockerell (Conchaspididae) (Site M'Bouzi). (F) Prédation d'une fleur de *V. humblotii*, avec des traces de morsures pouvant provenir d'un rongeur. (Photos R. Gigant).

Par ailleurs, certains ravageurs (souvent introduits) sont des menaces directes sur les populations de *V. humblotii* telle que la cochenille *Conchaspis angraeci* signalée dans les cultures du vanillier cultivé (*V. planifolia*) en 2003 (Richard 2003), qui s'attaque également aux populations de l'espèce sauvage et provoque l'affaiblissement de la liane et éventuellement sa mort (Figure 11E). De plus, certaines

fleurs de *V. humblotii* sont consommées, probablement par des rongeurs introduits, ce qui nuit inévitablement à la reproduction naturelle de l'espèce (Figure 11F).

***Vanilla roscheri*, espèce aphylle à fleur blanche endémique des îles et du littoral sud-est africain**

L'espèce aphylle *V. roscheri* est décrite comme possédant des tiges épaisses d'un brun rougeâtre de 12 à 20 mm de diamètre (Portères 1954b). Comme pour *V. humblotii,* les bractées sont visibles sur les jeunes pousses mais disparaissent rapidement. Les boutons floraux sont brunâtres, les fleurs épanouies blanches et les pétales elliptiques-oblongs, plus larges que les sépales (Portères 1954b). Le labelle ondulé sur les marges est rosée à l'intérieur avec deux lignes pubérulentes s'étendant vers la base. La capsule linéaire mesure jusqu'à 17,5 cm de long. L'espèce a été décrite principalement à partir de collectes réalisées au sud-est africain tropical, d'abord en Tanzanie (Dar-es-Salam) et sur les îles de Pemba et Zanzibar (Portères 1954b) puis au Mozambique (l'île Inhaca, (Krupko et al. 1954). Ce n'est que tardivement que l'espèce a été signalée en Afrique Du Sud, où *V. roscheri* a été identifiée sur les rives du lac Sibaya par Ward (1984) qui réalise pour la première fois des clichés de la fleur et témoigne de la fructification naturelle de l'espèce (Figure 12). Il s'agit des uniques données disponibles quant à la reproduction, la distribution de ce vanillier aphylle endémique du sud-est africain.

Au sein de la réserve d'iSimangaliso (KwaZulu Natal, Afrique Du Sud), cette espèce est classée deuxième orchidée la plus rare et menacée de la réserve, faisant donc l'objet d'une attention de conservation particulière par les autorités du Ezemvelo KwaZulu Natal Wildlife (EKZNW) (Combrink and Kyle 2006).

L'écosystème tropical qui abrite les populations de *V. roscheri* appartient à un vaste ensemble le long du littoral sud-est africain composé d'une mosaïque de forêt tropicale sèche et de fourrés secs, qui contient notamment le iSimangaliso Wetland Park classé patrimoine mondial par l'UNESCO, cinq sites RAMSAR (pour la protection des zones humides) et neufs Zones Importantes pour la

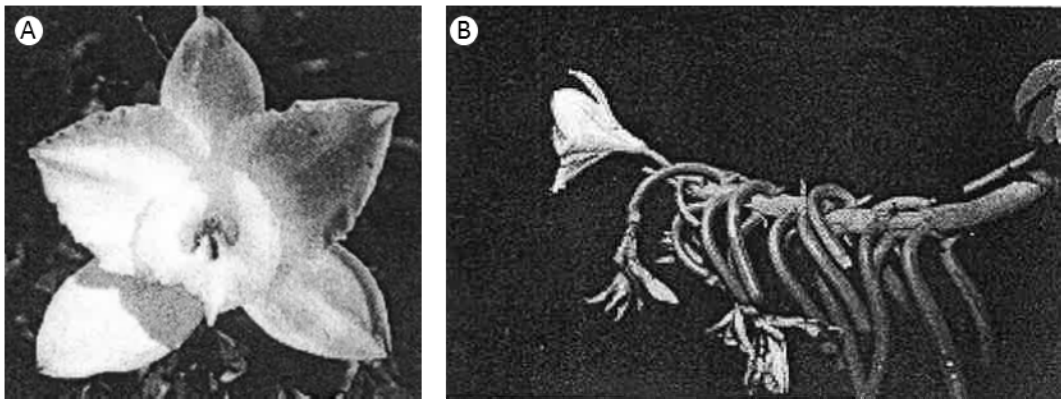

Figure 12 Clichés réalisés en 1984, lors de la découverte d'une population de *V. roscheri* en Afrique Du Sud près du Lac Sibaya avec la fleur de *V. roscheri* (A) et une inflorescence ayant fructifié (B) (Ward, 1984).

Conservation des Oiseaux (ZICO ou Important Bird Area) (Smith et al. 2008; Smith and Leader-Williams 2006).

La conservation des habitats naturels de la région doit faire face à l'implantation des infrastructures qui accompagne l'augmentation démographique des populations et favorise la surexploitation de l'environnement (bois de construction, bois de chauffe, plantes médicinales), l'extension de l'agriculture, auxquelles s'ajoutent les invasions biologiques et le développement des activités touristiques sur le littoral (Von Maltitz et al. 2003; Smith and Leader-Williams 2006).

La figure 13 met en évidence les zones protégées à proximité du lac Sibaya où se trouvent les populations de *V. roscheri* en Afrique Du Sud. Près de Sibaya, les aires protégées recouvrent essentiellement la bande de dune qui sépare le lac de l'océan Indien mais concernent aussi et seulement la zone immergée du lac (En bleu, Figure 13). Les rangers de l'EKZNW (Figure 14A) sont effectivement très impliqués dans la protection des vertébrés tels que l'hippopotame (*Hippopotamus amphibius* L. Hippopotamidae) (Figure 14B), les reptiles (Figure 14C), et les oiseaux, pour ne citer qu'eux. En revanche, les berges du lac composées de végétation tropicale sèche (Figure 14D) où se trouve *V. roscheri* ne sont pas concernées par les mesures de protection.

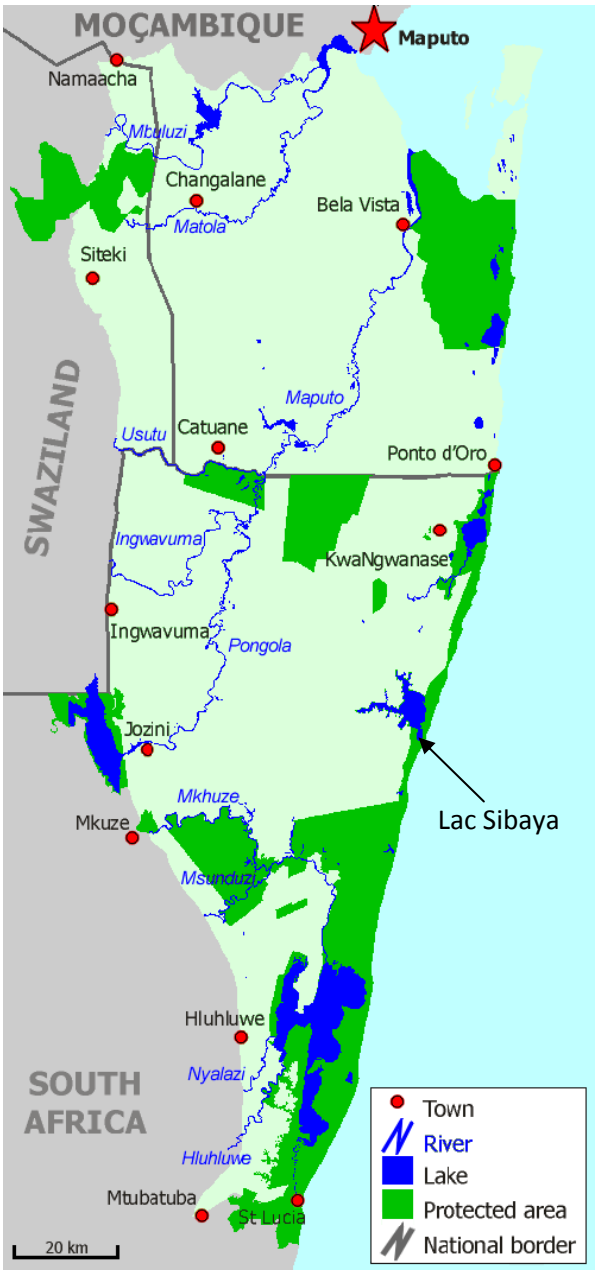

Figure 13 Distribution des zones protégées (vert foncé) dans la zone transfrontalière du Maputaland hébergeant le lac Sibaya (Smith and Leader-Williams 2006)

Or, ces zones sont l'objet d'une exploitation par les populations locales, qui menace directement ou indirectement les habitats naturels de *V. roscheri* bordant le lac. Le lac est effectivement la principale ressource en eau des villages, et est aussi exploité pour la pêche (Figure 15A). Ses abords sont également utilisés pour l'élevage de zébu (Figure 15B) et le bois des forêts pour les constructions mais aussi en pharmacopée traditionnelle (Figure 15C).

Figure 14 (A) Les rangers de l'EKZNW et les symboles de leur activité de lutte contre le braconnage: (B) l'hippopotame (*Hippopotamus amphibius* L.) (C) et le crocodile (*Crocodilus niloticus* Laurenti, ici, pris dans un piège de braconnier). (D) une berge du lac Sibaya (photos R. Gigant).

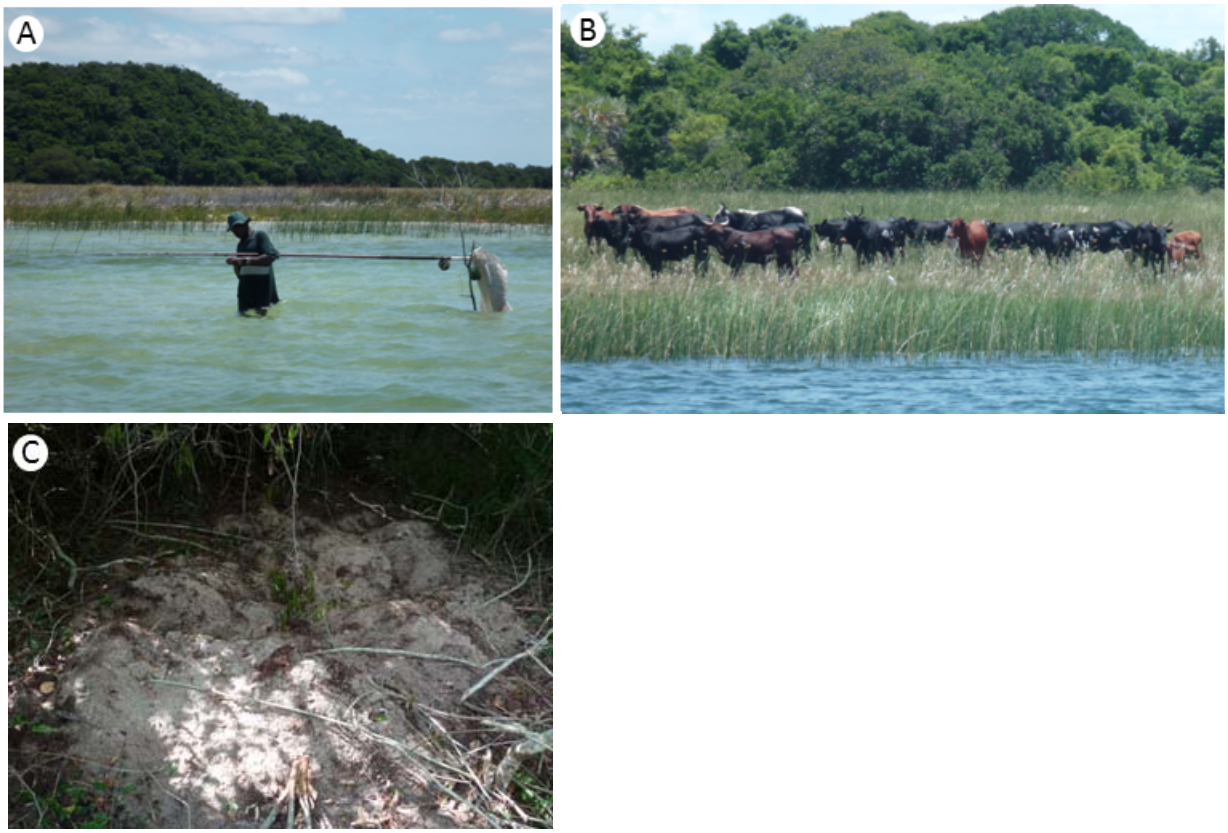

Figure 15 Quelques exemples d'exploitation des ressources du lac Sibaya. Pèche (A), élevage de zébus (B), et déforestation pour les usages en pharmacopée traditionnelle (C) sont autant de menaces sur les habitats des populations de *V. roscheri.* (Photos R. Gigant).

1.4 Objectifs de recherche de la thèse

Au regard des enjeux majeurs liés à la nécessité de préserver les ressources sauvages menacées des deux vanilliers de l'océan Indien *V. humblotii* et *V. roscheri*, les priorités de recherche dans le cadre de mon travail de thèse ont été:

Identifier les populations

- Identifier, recenser, géolocaliser et échantillonner avec précision les populations des espèces concernées.

- Commencer un échantillonnage des populations en vue de conserver *ex situ* (CRB Vatel) quelques individus par population, en vue de leur réintroduction si nécessaire.

Génotyper les populations

A l'aide d'outils moléculaires performants à l'échelle populationnelle, déterminer les niveaux de diversité et la structure génétique des populations de ces vanilliers sauvages.

Caractériser la reproduction / l'écologie de la pollinisation des espèces

-Déterminer quels sont les modes et les régimes de reproduction des espèces, et quelles sont leurs influences sur la structuration génétique et spatiale des populations ?

Des observations ponctuelles ont fait état de fructification naturelle de ces espèces. Si les espèces sont allogames comme la majorité des vanilliers, quels sont les pollinisateurs et quel est le taux de fructification naturel de ces espèces? Les visiteurs des fleurs sont-ils d'efficaces pollinisateurs?

Les variations dans les morphologies florales se traduisent-elles par des interactions avec différents pollinisateurs ?

Proposer des mesures de conservation

Que nous enseignent les études écologique et génétique des espèces en termes de conservation ? Quelles sont les mesures de conservation, *in situ* et ex *situ,* que nous pouvons préconiser au regard des analyses des systèmes de reproduction et de la diversité génétique des populations ?

Le chapitre 2 de cette thèse présentera brièvement la stratégie d'étude et le développement des marqueurs microsatellites adaptés à nos questions de recherche dans un article publié. Le chapitre 3 présentera sous forme de deux articles scientifiques les résultats des études génétiques et de biologie de la reproduction à l'échelle locale et régionale de l'espèce *V. humblotii.* Le quatrième chapitre rendra compte sous forme d'un article scientifique, des résultats de génétique des populations et de biologie de la reproduction obtenus sur l'espèce *V. roscheri* en Afrique Du Sud.

Le chapitre 5 sera l'occasion de comparer les résultats obtenus à partir des deux modèles étudiés. Nous mettrons l'accent sur la complémentarité et l'intérêt des approches employées pour la conservation, et nous tenterons de généraliser les résultats à l'ensemble des espèces aphylles de l'océan Indien.

CHAPITRE 2

CHAPITRE 2 : Stratégie d'étude et méthodes

Afin de répondre à la problématique liée à la conservation de *V. humblotii* et de *V. roscheri*, la première étape a été de mettre en place des collaborations scientifiques et techniques pour l'identification et le suivi des populations, puis de réaliser des missions d'échantillonnage et d'étude.

2-1 Les collaborations

***Vanilla humblotii* à Mayotte**

Pour l'étude des populations de *V. humblotii* à Mayotte, des collaborations ont été développées avec le Conservatoire Botanique de Mascarin de La Réunion (Les Colimaçons, Saint-Leu) qui possède aussi une antenne spécifique à Mayotte. Nous avons également développé une collaboration avec la Direction de l'Agriculture et des Forêts de Mayotte. Leur appui technique a été un atout considérable pour l'identification des habitats potentiels de *V. humblotii* à Mayotte.

- Une mission exploratoire a pu être réalisée du 01 Août au 10 Août 2009 afin d'identifier les populations de *V. humblotii.* L'objectif était d'échantillonner le plus grand nombre de populations, afin d'avoir une représentation fidèle de la diversité génétique totale de l'espèce à l'échelle de l'île.
- Une mission d'échantillonnage exhaustif des larges populations et complémentaire à la première mission a été réalisée du 25 Novembre au 19 Décembre 2010. Cette mission réalisée pendant la période de floraison de *V. humblotii* avait pour but de compléter l'échantillonnage de la première mission au sein de larges populations, d'en collecter de nouvelles afin de comparer la diversité génétique des populations et d'étudier la structure génétique spatiale à petite échelle. De plus, la biologie de la reproduction, les systèmes d'incompatibilités de la reproduction, l'écologie de la pollinisation ont également pu être étudiés en conditions naturelles.

***Vanilla roscheri* en Afrique Du Sud**

Notre projet a fait l'objet d'une convention de recherche avec les autorités en charge du iSimangaliso Wetland Park et le Ezemvelo KwaZulu Natal Wildlife et leur appui technique et logistique pour l'identification des populations, a été un atout majeur pour la conduite de nos recherches :

- une mission exploratoire a été réalisée en Mai 2009 (Pr. Pascale Besse) afin d'identifier des populations et de collecter une première série d'échantillons pour les analyses génétiques.

- deux missions d'échantillonnage en période de floraison ont ensuite été réalisées du 18 au 27 Janvier 2010 et du 14 Janvier au 02 Février 2011 afin de poursuivre les prospections des populations de *V. roscheri* sur les rives du lac Sibaya et de réaliser des expérimentations *in situ* en biologie de la reproduction (systèmes d'incompatibilité de la reproduction et écologie de la pollinisation).

2.2 Développement des marqueurs microsatellites

Afin de caractériser la diversité génétique des populations des deux vanilliers aphylles, des marqueurs moléculaires microsatellites, hautement polymorphes, ont été développés spécifiquement à partir de ces espèces. Grâce à cet outil, des analyses de la structure spatiale génétique à petite et grande échelles ont également pu être menées. Ces données ont été ensuite couplées aux analyses de la biologie de la reproduction et de l'écologie de la pollinisation.

Nous présenterons dans l'article de recherche suivant, la méthode et les résultats du développement de la banque de marqueurs microsatellites.

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TECHNICAL NOTE

Nineteen polymorphic microsatellite markers from two African *Vanilla* species: across-species transferability and diversity in a wild population of *V. humblotii* from Mayotte

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Abstract There is a serious lack of information on the genetic diversity and population dynamics of the 110 tropical *Vanilla* species, although these are keys elements to adjust conservation strategies. Nineteen polymorphic microsatellite markers were developed from two African leafless *Vanilla* species *V. roscheri* and *V. humblotii* to use in population genetic studies. A transferability analysis of these markers on seven *Vanilla* species from various geographical origins was conducted. Nine microsatellites were polymorphic in a population of 22 individuals of *V. humblotii* from Mayotte (Comoros Archipelago). These markers had two to eight alleles per locus and observed heterozygosity ranged from 0.23 to 0.64. Amplification parameters were calibrated to facilitate multiplexing and rapid multi-loci genotyping.

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Keywords Conservation genetics · *Vanilla* · Microsatellite · Orchids

The *Vanilla* Plum. ex Miller genus (Orchidaceae family) contains about 110 species distributed throughout the tropics (Portères 1954). Most species are hemi-epiphytic leafy vines growing in tropical wet forests (Portères 1954), but adaptation to xeric conditions resulted in 18 paraphyletic leafless species found in tropical dry forests (Bouetard et al. 2010). Seven of these species are distributed in the Southwest Indian Ocean (SWIO) area (Portères 1954). Taking the high biodiversity value of this region into consideration (Myers et al. 2000) together with the fact that tropical dry forests are considered amongst the most threatened forests in the world (Hoekstra et al. 2005; Janzen 1988), these orchids deserve high conservation priority. Preservation guidelines will necessitate the development of genetic markers for population genetic studies to unravel the mechanisms of maintenance and dynamics of *Vanilla* populations (Gigant et al. 2011). Bory et al. (2008) developed a first set of 14 microsatellites from the American species *V. planifolia,* but only two were transferable to African species (mVplCIR025 and mVplCIR031). Therefore two microsatellite libraries were developed from two African leafless species, *V. humblotii* Rchb. f., and *V. roscheri* Rchb. f..

DNA extractions following the CTAB protocol (Risterucci et al. 2000) were made from CR0108 (*V. humblotii*) and CR0809 (*V. roscheri*), collected in the Biological Resource Center (BRC) Vatel located in Saint-Pierre, Réunion Island (Roux-Cuvelier and Grisoni 2010). We constructed a genomic enriched library (TGn and TCn) for each species following the protocol of Billote et al. (1999)

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Table 1 Transferability of nineteen nuclear microsatellites (primer pair sequences F, forward; R, reverse) in a set of *Vanilla* species

Locus name	Repeat motif	Primer sequence (5'–3')	Dye label	American species																GenBank
				V. planifolia (N = 4)				*V. pompona* Schiede (N = 4)				*V. palmarum* Lindl. (N = 2)				*V. bahiana* Hoehne (N = 4)				
				Sr	P	N_a	AI	Sr	P	N_a	AI	Sr	P	N_a	AI	Sr	P	N_a	AI	
mVroCIR01	(ga) 13	F: GCTTGCAAAGACGAGAAAGG	FAM	123–148	NP	2	4	189–194	P	6	4	132–140	P	3	2	125–155	P	7	4	JN222572
		R: CTCGATCCTCACACCTGCTT																		
mVroCIR03	(ga) 16	F: AAGAAGGCTTAGAGATTGACAACAA	FAM	293–344	NP	2	4	293–351	P	3	4	293–347	P	2	2	293–347	P	2	3	JN222573
		R: TCCAGTTCAAAGGAGGTTGA																		
mVroCIR04	(ga) 18	F: AAGGTACAAGATCCCCGTCA	PET	204–210	P	3	2	193–200	NP	2	2	200–206	NP	2	1	188–206	P	4	3	JN222574
		R: CCGCGAGCTTTATTCTACCA																		
mVroCIR05	(ga) 15	F: GCTATTTCCCACGAACCCTA	NED	196	NP	1	4	194	NP	1	4	190–198	P	3	2	198–204	P	3	4	JN222575
		R: AACCATTGCCAGAAGCCTAA																		
mVroCIR06	(tg) 9	F: CCCGTCTAAGGGGCTAGTGA	FAM	177–195	P	3	4	179–209	P	4	3	189–195	P	2	2	189–224	P	3	4	JN222576
		R: GGAAAATCAAAGGCATTACACC																		
mVroCIR08	(ag) 16	F: CATCGTCATCTTCGGGATCT	VIC	200–242	P	3	4	184–209	P	4	4	184–195	P	3	2	201–209	P	3	4	JN222577
		R: CCATCCTCAAAACCATCTCG																		
mVroCIR09	(ga) 19	F: CAAAATGCCAAGATGCAGAA	PET	–	–	–	0	–	–	–	0	212–237	P	4	2	–	–	–	0	JN222578
		R: TCGGTGACGAATACAAGGTTT																		
mVroCIR10	(ga) 15	F: CAAGTCCCCTTCTTGGTTGA	VIC	189–223	P	4	4	189–212	P	2	4	212–216	P	3	2	216–236	P	3	4	JN222579
		R: CCCATGTGCGTTGTCTTATT																		
mVroCIR11	(ga) 15	F: CCCGCGAGCCTATATTTGT	PET	160–200	P	3	3	170–195	P	3	4	184–187	P	2	2	168–184	P	4	4	JN222580
		R: CCATCCTGCTGCAATCGT																		
mVhuCIR03	(ag) 24	F: GTCGCATTCACATAGCTTCG	FAM	124–127	P	2	4	124–127	P	2	4	124	NP	1	2	124–138	P	2	4	JN222562
		R: TCTTCCTACCGCTGTCGTCT																		
mVhuCIR04	(ag) 13	F: GGATACTTCCGGTGACTCCA	FAM	153–160	P	2	4	158–191	P	6	4	129–158	P	2	2	134–170	P	6	4	JN222563
		R: GCTCTGGCTCTGTGGTTAGG																		
mVhuCIR06	(tg) 10	F: CTTGGTTGTTCTGCTGGATG	PET	–	–	–	0	–	–	–	0	–	–	–	0	–	–	–	0	JN222564
		R: CCCTAGAACCCATGTCTTGC																		
mVhuCIR07	(ga) 16	F: CCACGTAGATCAAACACAGCA	NED	170–190	P	4	4	174–238	P	4	4	174–204	P	4	2	170–232	P	6	4	JN222565
		R: AAGAAACTGTTAGAAATCCCAAGC																		
mVhuCIR08	(ga) 15	F: TTAACTCTTCGCCCATTAAGC	FAM	230–244	P	2	4	200–224	P	3	4	214–248	P	4	2	232–242	P	4	4	JN222566
		R: CCTTCGAGCTTTCCGTTC																		
mVhuCIR09	(ag) 14	F: CGCCGGATACTTCCAATTAC	VIC	–	–	–	0	–	–	–	0	186–190	P	2	2	–	–	–	0	JN222567
		R: TGATTCACGCATGTAAACGAG																		
mVhuCIR10	(ca) 5	F: AGCTCTCCGAATCTCCATCC	FAM	198–212	P	3	4	192–204	P	2	4	192–204	P	4	2	196–210	NP	2	4	JN222568
		R: GGGAACATGAAACAGGAAGC																		
mVhuCIR11	(tg) 9	F: TTTGACAAGTTGCATTGGTTG	VIC	241–266	P	3	4	239	NP	2	2	239–241	P	2	2	241	NP	1	2	JN222569
		R: GGCGTCTAAGGAAACTGCAC																		
mVhuCIR12	(tc) 11	F: AAATATCAAGCACAATCCATTCC	FAM	225–231	P	2	2	–	–	–	0	221–233	P	3	2	223–225	NP	2	1	JN222570
		R: TCGGCATGTTCTCATGTTTT																		
mVhuCIR14	(ga) 8	F: AGAAAGATGCCCAAAAGCAA	NED	215	NP	1	1	–	–	–	0	–	–	–	0	213	NP	1	2	JN222571
		R: CTGAGACTTCAACCATCGTCA																		

Table 1 continued

Locus name	Repeat motif	Primer sequence (5'–3')	Dye label	African species												GenBank
				V. imperialis Kraenzl. (N = 3)				*V. madagascariensis* Rolfe (N = 7)				*V humblotii* (CA) (N = 37)				
				Sr	P	N_a	AI	Sr	P	N_a	AI	Sr	P	N_a	AI	
mVroCIR01	(ga) 13	F: GCTTGCAAAGACGAGAAAGG R: CTCGATCCTCACACCTGCTT	FAM	113–148	P	3	3	125–160	P	5	7	122	NP	1	37	JN222572
mVroCIR03	(ga) 16	F: AAGAAGGCTTAGAGATTGACAACAA R: TCCAGTTCAAAGGAGGTTGA	FAM	229–367	P	3	3	307–353	P	5	7	364–380	P	8	36	JN222573
mVroCIR04	(ga) 18	F: AAGGTACAAGATCCCCGTCA R: CCGCGAGCTTTATTCTACCA	PET	184–204	P	3	3	200–212	P	4	7	198–212	P	4	37	JN222574
mVroCIR05	(ga) 15	F: GCTATTTCCCACGAACCCTA R: AACCATTGCCAGAAGCCTAA	NED	190–203	P	4	3	188–205	P	5	7	190–198	P	4	37	JN222575
mVroCIR06	(tg) 9	F: CCCGTCTAAGGGGCTAGTGA R: GGAAAATCAAAGGCATTACACC	FAM	189–259	P	5	3	174–196	P	4	7	–	–	–	0	JN222576
mVroCIR08	(ag) 16	F: CATCGTCATCTTCGGGATCT R: CCATCCTCAAAACCATCTCG	VIC	200–209	P	4	3	167–201	P	5	7	184	NP	1	37	JN222577
mVroCIR09	(ga) 19	F: CAAAATGCCAAGATGCAGAA R: TCGGTGACGAATACAAGGTTT	PET	203	NP	1	2	211–265	P	6	7	229–237	P	3	37	JN222578
mVroCIR10	(ga) 15	F: CAAGTCCCCTTCTTGGTTGA R: CCCATGTGCGTTGTCTTATT	VIC	189–210	P	3	3	197–213	P	6	7	210–214	P	3	37	JN222579
mVroCIR11	(ga) 15	F: CCCGCGAGCCTATATTTGT R: CCATCCTGCTGCAATCGT	PET	160–184	P	2	3	160–218	P	6	7	–	–	–	0	JN222580
mVhuCIR03	(ag) 24	F: GTCGCATTCACATAGCTTCG R: TCTTCCTACCGCTGTCGTCT	FAM	124–142	P	4	3	127	NP	1	7	149–159	P	5	37	JN222562
mVhuCIR04	(ag) 13	F: GGATACTTCCGGTGACTCCA R: GCTCTGGCTCTGTGGTTAGG	FAM	153–205	P	4	3	158–178	P	4	7	150–156	P	2	37	JN222563
mVhuCIR06	(tg) 10	F: CTTGGTTGTTCTGCTGGATG R: CCCTAGAACCCATGTCTTGC	PET	–	–	–	0	265–272	P	3	3	254–260	P	2	37	JN222564
mVhuCIR07	(ga) 16	F: CCACGTAGATCAAACACAGCA R: AAGAAACTGTTAGAAATCCCAAGC	NED	168–216	P	3	3	170–202	P	5	7	181–189	P	3	37	JN222565
mVhuCIR08	(ga) 15	F: TTAACTCTTCGCCCATTAAGC R: CCTTCGAGCTTTCCGTTC	FAM	213–230	P	2	3	240–254	P	4	7	216	NP	1	37	JN222566
mVhuCIR09	(ag) 14	F: CGCCGGATACTTCCAATTAC R: TGATTCACGCATGTAAACGAG	VIC	176	NP	2	3	178–218	P	7	7	190–210	P	6	37	JN222567
mVhuCIR10	(ca) 5	F: AGCTCTCCGAATCTCCATCC R: GGGAACATGAAACAGGAAGC	FAM	200–216	P	4	3	202–216	NP	2	7	216	NP	1	37	JN222568
mVhuCIR11	(tg) 9	F: TTTGACAAGTTGCATTGGTTG R: GGCGTCTAAGGAAACTGCAC	VIC	236–257	P	4	3	255–268	P	5	7	252–270	P	2	37	JN222569
mVhuCIR12	(tc) 11	F: AAATATCAAGCACAATCCATTCC R: TCGGCATGTTCTCATGTTTT	FAM	221–229	P	2	2	225–239	P	3	7	234	NP	1	37	JN222570

Table 1 continued

Locus name	Repeat motif	Primer sequence (5'–3')	Dye label	African species				*V. madagascariensis* Rolfe (N = 7)				*V. humblotii* (CA) (N = 37)				GenBank
				V. imperialis Kraenzl. (N = 3)												
				Sr	P	N_a	AI	Sr	P	N_a	AI	Sr	P	N_a	AI	
mVhuCIR14	(ga) 8	F: AGAAAGATGCCCAAAAGCAA R: CTGAGACTTCAACCATCGTCA	NED	213–215	P	2	3	213–215	P	2	6	216	NP	1	37	JN222571

In the locus name Vro indicates that the markers were developed from *V. roscheri* and Vhu from *V. humblotii*

Number of individuals (N), allele size range (Sr), polymorphic (P) or non polymorphic (NP) profile, number of alleles (N_a), number of amplified individuals (AI), sampling at the Comoros Archipelago scale (CA)

with slight modifications (1) the first step of DNA restriction was performed using *Hae* III (Biolabs), (2) DH10B supercompetent cells (Invitrogen) were used, (3) recombinant clones were screened by PCR with *Rsa* primers (*Rsa*21: 5′-CTCTTGCTTACGCGTGGACTA-3′; *Rsa*25: 5′-TAGT CCACGCGTAAGCAAGAGCACA-3′). A total of 192 recombinant colonies (96 for each library) were PCR-amplified for 35 cycles. After a transfer onto nylon membranes (Hybond N+, Amersham) and a screen for microsatellites with 32P radiolabelled synthetic microsatellite probes, 69 positive inserts were selected and sent for sequencing (Cogenics). The SAT software (Dereeper et al. 2007) (http://sat.cirad.fr/sat/) was used to check and align SSR motives. Twenty-five microsatellite loci with at least six repeat units and suitable flanking regions were selected and primers were designed. Following suitable amplification patterns on CR0108 and CR0809, 19 primer pairs were selected and amplifications were extended to a larger set of *Vanilla* species (Table 1). PCR was performed in a final volume of 15 µL containing: e7.5 µL of Qiagen Multiplex PCR Master Mix (2×), 1.4 µL of Qiagen Q-solution (5×), 0.3 µM of each reverse primer, 0.3 µM of each forward primer end-labelled with a fluorescent dye (Table 1), and about 25 ng of DNA template. The PCR multiplex conditions were as follows: denaturation at 94°C for 4 min, 40 cycles at 94°C for 1 min, 57°C for 30 s, 72°C for 1 min, and final elongation at 72°C for 7 min. The annealing temperature was exactly the same for all the primers (57°C) which facilitated multiplexing. Analyses of PCR products including an internal size standard (Genescan LIZ 500, Applied Biosystems) were performed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and allele sizes were estimated using Genemapper 3.7 software (Applied Biosystems).

Cross amplification of the 19 microsatellites was tested on seven *Vanilla* species from the BRC Vatel (Table 1). A high success of cross-amplification in divergent African and American species (Bouetard et al. 2010; Soto Arenas and Cameron 2003; Soto Arenas and Cribb 2010) was revealed with a mean of 16.7 amplified loci per species.

A detailed study of *V. humblotii* at the Comoros Archipelago scale revealed 11 polymorphic markers (Table 1). Of these, nine were polymorphic on 22 *V. humblotii* individuals from a wild population in Mayotte Island from a single location (Table 2). Number of alleles, observed and expected heterozygosity were calculated using GenAlex 6.41 software (Peakall and Smouse 2006), and null allele frequency were estimated by Microchecker 2.2.3 (Van Oosterhout et al. 2004; Chapuis and Estoup 2007). Tests for deviations from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium were conducted using Genepop 4.0 (Raymond and Rousset 1995) (Table 2). The nine microsatellite loci produced 31 alleles in total. Number of

Tableau 8

Table 2 Genetic characteristics in a *V. humblotii*'s population (N = 22) from Mayotte Island for the nine polymorphic microsatellite loci

Locus name	N_a	Size-range (bp)	H_o	H_e	F_{is}	r
mVroCIR03	6	364–376	0.50	0.64	0.22	0.08
mVroCIR04	3	198–210	0.59	0.58	−0.04	0.00
mVroCIR05	3	190–202	0.55	0.55	0.01	0.00
mVroCIR09	2	229–231	0.23	0.45	0.494*	0.15 (y)
mVhuCIR03	3	155–159	0.41	0.39	−0.04	0.00
mVhuCIR04	2	150–156	0.55	0.40	−0.38	0.00
mVhuCIR06	2	254–260	0.64	0.50	−0.28	0.00
mVhuCIR07	2	181–189	0.50	0.49	−0.02	0.00
mVhuCIR09	8	200–210	0.55	0.62	0.12	0.05

Number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{is}), exact null allele frequency (r), evidence for null allele (y). * Significant deviation from HWE was detected ($P < 0.05$)

allele per locus varied from 2 to 8 with a median of 3 alleles per locus. Expected and observed heterozygosity ranged from 0.39 to 0.64 and 0.23 to 0.64, respectively (Table 2). Significant linkage disequilibrium was detected in only 1 of 36 pairs of loci (VhuCIR04 and VhuCIR07) ($P < 0.05$). No deviation from HWE was detected, except for the locus VroCIR09 ($P < 0.05$) for which the deviation was attributed to the presence of null alleles. The multi-loci exact test performed by the Markov chain method suggested that the *V. humblotii* population complied with HWE.

In conclusion, the set of microsatellite markers developed from African *Vanilla* leafless species should significantly increase the possibilities of in-depth investigation of the genetic diversity in a wide range of *Vanilla* species to plan conservation actions.

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CHAPITRE 3

CHAPITRE 3 : Etude de *Vanilla humblotii* à Mayotte

3.1 Préambule

Le troisième chapitre est consacré à l'étude de la contribution des modes de reproduction (sexuée et asexuée) à la structuration spatiale de la diversité dans les populations naturelles de *V. humblotii* à l'échelle populationnelle (neuf populations ont été identifiées) et à l'échelle de Mayotte. Bien que l'espèce soit protégée par arrêté préfectoral, les pressions anthropiques sur les habitats naturels de l'espèce sont importantes. Actuellement, les forêts tropicales sèches ne se retrouvent en effet qu'en l'état de reliques, extrêmement fragmentées et isolées à Mayotte. Les connaissances de la biologie de la reproduction et l'étude de la diversité génétique des populations seront donc des éléments essentiels pour la conservation de l'espèce *V. humblotii*.

Ce chapitre s'articule autour de deux articles scientifiques couplant le développement des marqueurs microsatellites à l'étude de la biologie de la reproduction. L'influence de la fragmentation des habitats sur l'interaction entre diversité génétique et reproduction de *V. humblotii* est testée dans le but de suggérer des mesures appropriées pour la conservation du vanillier indigène à Mayotte.

Le premier article combine une étude approfondie de la biologie de la reproduction et une analyse de la structure génétique et spatiale à petite échelle dans la plus grande population identifiée (Sohoa). L'espèce nécessite effectivement de pollinisateurs pour se reproduire sexuellement, mais la structuration de la diversité à petite échelle est influencée aussi par la reproduction végétative. Deux visiteurs ont été observés, une abeille sauvage (*Allodape obscuripennis,* Xylocopinae) et un souimanga *(Nectarinia coquerelli*, Nectariniidae) mais aucun mouvement de pollen et un taux moyen de fructification très faible ont été enregistrés. La diversité génotypique reste cependant importante dans la population de Sohoa étudiée. La distribution de type phalanx de la clonalité explique les autocorrélations significatives à petite distance, mais l'augmentation attendue de la fréquence des autofécondations par geitonogamie, qui peut provoquer une consanguinisation et/ou de la dérive

génétique, reste limitée par la diversité génotypique élevée associée à une dispersion des graines supérieure à celle du pollen et probablement à une stratégie d'évitement de la geitonogamie.

Le deuxième article s'appuie sur un échantillonnage représentatif des populations de *V. humblotii* à l'échelle de Mayotte pour déterminer la structuration génétique des populations naturelles, au regard de leur importante fragmentation et de l'influence des barrières géographiques sur l'isolement des populations. Bien que certaines populations soient réduites à moins de 10 individus différents, les taux d'hétérozygotie et la diversité génotypique restent relativement élevés. Cependant ces populations ont subit d'importantes réductions d'effectifs, révélés à la fois par une perte de diversité allélique, et la fréquence élevée des allèles privés dans ces populations ce qui montre l'importance de considérer aussi la diversité génétique de ces petites populations. La majorité de la variance génétique se retrouve à l'intérieur des populations, ce qui reflète le régime allogame de l'espèce. Un patron d'isolement par la distance a été détecté entre populations rapprochées, mais des évènements de dispersion à longue distance nuancent la différentiation génétique attendue lors de la présence de barrières géographiques. La longévité de *V. humblotii*, associée à la multiplication végétative de l'espèce et au caractère récent de la fragmentation de l'habitat, ont probablement maintenu les génotypes, et limité les conséquences de la consanguinisation et de la dérive génétique attendues suite à l'isolement des populations. Toutefois, en l'absence de reproduction sexuée et au rythme soutenu de la déforestation, la survie à long-terme des populations naturelles est remise en question. En conclusion, des propositions de gestion *in situ* et *ex situ* des populations sont avancées pour la conservation de *V. humblotii* à Mayotte.

3.2 Article 2

Reproductive strategies and their consequences on fine-scale spatial genetic structure and diversity of the indigenous and endangered leafless *Vanilla humblotii* Rchb. f. (Orchidaceae) from Mayotte (Comoros Archipelago, Indian Ocean)

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3.2.1 Abstract

Background In *Vanilla species* (Orchidaceae), the contribution of both sexual and asexual mating systems on the spatial structuring of diversity in populations is understudied although these elements are crucial for conservation biology. *Vanilla humblotii* is a wild endangered leafless species described locally abundant in the remnant fragments of tropical dry forest in Mayotte (Comoros Archipelago, Indian Ocean).

Methods Approaches combining reproductive biology and Fine-scale Spatial Genetic Structure analyses of *V. humblotii* are employed to unravel the reproductive strategies of the species.

Results The species was allogamous and also autocompatible with a pollinator-dependent system. A total absence of pollen movements and a low level of natural fruit set (0.8%) were reported, although a wild bee (*Allodape obscuripennis*, Xylocopinae) and a bird (*Nectarinia coquerelli,* Nectarinidae) visited the flowers. A high genotypic diversity (0.88) and a phalanx distribution of the clonality were detected, and seed dispersal was higher than pollen dispersal.

Conclusions The phalanx distribution of the clonality is responsible for significant autocorrelations detected at small distances. However, the high genotypic diversity, related to a pollinator-dependent system and seed dispersal ability, limited the impacts of inbreeding through geitonogamy which could have been enhanced by vegetative reproduction. The low fruit set is related to the absence of pollen movements and probably linked with the hostile surrounding degraded landscape preventing plant-pollinator interactions.

Keywords: mating systems, pollinator, Fine-scale Spatial Genetic Structure, clonality, *Vanilla humblotii*

3.2.2 Introduction

Vanilla Plum. ex Miller genus is a monophyletic primitive lineage of the Orchidaceae family (Cameron 2004, 2005) that contains over 100 species widely distributed throughout the tropics in America, Asia and Africa (Portères 1954). Reproduction in most *Vanilla* species is described as a mixed mating system combining a predominant a vegetative propagation due to natural stem cuttings of the semi-hemiphytic vines and a pollinator mediated sexual reproduction (Bory et al. 2010; Gigant et al. 2011a). Most *Vanilla* species flowers exhibit an efficient rostellum resulting in a pollinator-dependant system for sexual reproduction (Bory et al. 2008c; Ackerman 1983; Bouriquet 1954; Dobat and Peikert-Holle 1985; Gigant et al. 2011a; Soto Arenas 1999a; Soto Arenas and Cameron 2003; Stéhlé 1954; Soto Arenas and Dressler 2010). Only few species are spontaneous self fertilisers due to a reduced rostellum or stigmatic leak (Gigant et al. 2011a) and display high (up to 78%) natural fruit-set (Gigant et al. 2011a). In other species, sexual reproduction involves an insect-dependant system for pollination, although other pollinators such as hummingbirds are also suspected, with natural fruit set ranging from 0.1% to 18.2%, probably dependant of pollinator availability, as reviewed in Gigant et al. (2011a). So far, pollinators identified for *Vanilla* species concern only some American *Vanilla* species and involve mainly euglossine orchid bees (Soto Arenas and Dressler 2010; Lubinsky et al. 2006; Householder et al. 2010; Soto Arenas 1999c; Soto Arenas and Cameron 2003; Bory et al. 2008a; Gigant et al. 2011a). Due to nectarless flowers in the studied *Vanilla* species a 'male euglossine syndrome' (Williams and Whitten 1983; Bembé 2004), was hypothesized for *Vanilla* species with fragrant flowers, where male bees collect fragrance compounds then stored in swollen glandular tibiae of their rear legs (Dodson et al. 1969) and indirectly achieve pollination. Pollination involving euglossine bees were shown in at least two species, *V. trigonogarpa* by *Euglossa asarophora* (Soto Arenas and Dressler 2010) and *V. pompona* subsp. *grandiflora* by *Eulaema meriana* (Lubinsky et al. 2006) but were not associated with a fragrance collection behavior. Most of *Vanilla* species are more likely pollinated by a deceptive system (Soto Arenas 1999c; Soto Arenas and Cameron 2003; Gigant et al. 2011a) as are approximately a third of known orchids species (Jersáková

et al. 2006; Schiestl 2005; Singer 2003; Tremblay et al. 2005). Another pollination syndrome was also proposed for V. *inodora* involving large carpenter bees (*Xylocopa sp.*) (Soto Arenas and Cameron 2003). For Asian species, the only reported pollinator is a large Aegilopa bee for the species *V. cf kaniensis* in Papua New Guinea (Soto Arenas and Cameron 2003). In Africa, nothing is known about the reproductive system of any *Vanilla* species (Van der Cingel 2001). *V. humblotii* Rchb. f is a leafless *Vanilla* species found in the South West Indian Ocean (SWIO) area in the Comoros Archipelago (Mayotte, Moheli, Anjouan, Grande Comore) and also possibly in Madagascar (Cribb and Hermans 2009; Lecoufle and Bosser 2011; Portères 1954). In Mayotte, the species is scarce and protected since 2006 (Arrêté Préfectoral APn° 42/DAF/2006 3rd may 2006) but its status is uncertain on the other islands of the archipelago where it might be on the edge of extinction (L. Gigord, com. pers.). Defining a conservation plan for this species is therefore considered as a priority, and requires prior knowledge on its reproductive biology. At a fine scale, the spatial structure of genotypes allows learning more about both the nature and the relative importance of different breeding strategies, i.e. the influence of pollen and seed dispersal as well as the structure caused by spread of vegetative clones (Epperson and Allard 1989; Vekemans and Hardy 2004; Hamrick and Trapnell 2011; Alberto et al. 2005; Vallejo-Marín et al. 2010; Debout et al. 2011). We therefore conducted both *in situ* observations and reproductive biology experiments in Mayotte, which were combined with a molecular genetic diversity study on the largest remnant population (Sohoa) using previously developed microsatellite markers (Gigant et al. 2011b; Bory et al. 2008b), to unravel the reproductive strategy in *V. humblotii* and its consequences on the genetic diversity and its spatial structuring.

3.2.3 Materials and Methods

Study site

The Comoros Archipelago is composed of four main islands (Mayotte, Moheli, Anjouan, Grande Comore) emerged from the drift of the Somali plate on top of a hot spot plume (Emerick and Duncan 1982; Späth A. et al. 1996) and located in the northern part of the Mozambique Channel between

Madagascar and Mozambique. The French Overseas Territory Mayotte (379 km²) is the southernmost (15° 33' S, 54° 31' E) and oldest island of the archipelago (around -10 Myrs) culminating at 660 m at the Benara Peak. The strong volcanic geomorphology is still visible by old craters and an undulating topography of elongated hills formed by lava flows (Audru et al. 2010). Primary natural forests have become rare in Mayotte and persist mainly on hilltops. The conservation of these forest patches is a priority given that the majority of the archipelago's floral richness in native and endemic plants is concentrated in these remnant forests and distributed in only 15 km² of the whole land area of Mayotte (Vos 2004).

The Sohoa forest reserve covers 208 ha with an average elevation of 190 m (Figure 1). Sohoa is mainly composed uphill by a rainforest and by a fragment of mesophilous forest extending down to the sea, in which we found a large population of *Vanilla humblotii*. The mesophilous forest is the least preserved of all natural formations in Mayotte, covering nowadays only 85 ha in Mayotte and reduced to two relict fragments in Sohoa and Dapani (Pascal et al. 2001; Laybourne 2010). This plant formation used to be much more extended before the intense lowland clearings in the nineteenth century for agriculture (sugarcane, coffee and cocoa) (Pascal et al. 2001; Guéneau 2006). Although selective logging lasted until the nineties in the Sohoa reserve, Euphorbiaceae (*Drypetes comorensis* (Baill.)) and Sapotaceae (*Mimusops comorensis* Engl. and *Chrysophyllum boivinianum* (Pierre) Baehni) are particularly abundant, forming a tree stratum of 15-20 m heigh (Pascal et al. 2001). Pandanaceae (*Pandanus mayotteensis* H. St. John and *P. associatus* Huynh) and Violaceae (*Rinorea spinosa* (Tul.) Baill. are typical of the brushwood layer and seedlings and ferns compose the essential of the herbaceous plants (Pascal et al. 2001).

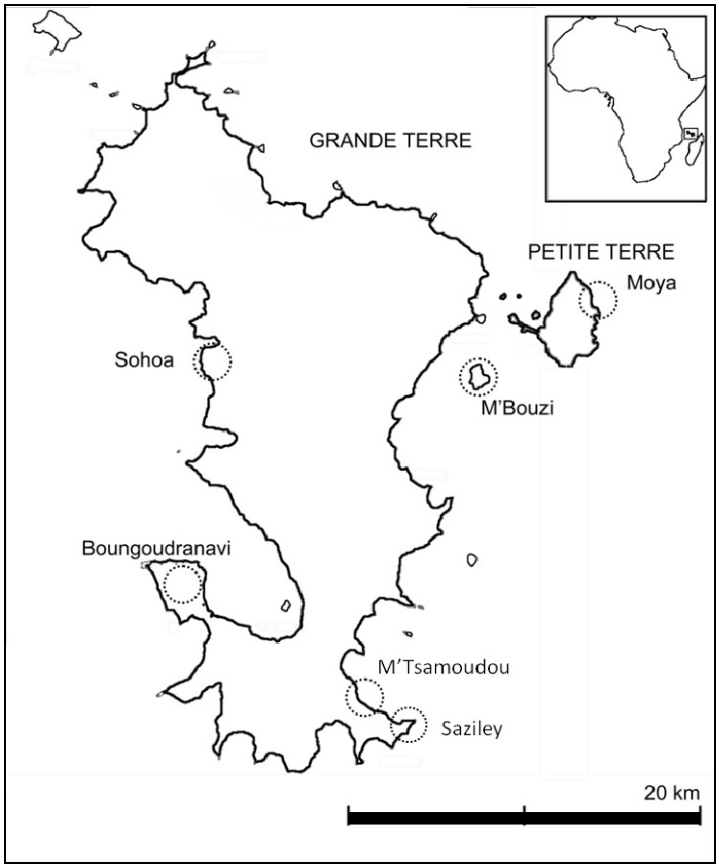

Figure 16 - Figure 1 Map of Mayotte showing the study sites of *V. humblotii.*

Study species

Belonging to the genus *Vanilla* (Vanilloideae sub-family, Vanilleae tribe and Vanillinae sub-tribe), *V. humblotii* is a leafless hemi-epiphytic vine with an important ecological plasticity, found from 0-600 m altitude both in extremely dry conditions on rocky environments (Portères 1954) and in mesophilous forest. The stem is large and glaucescent (Portères 1954) with occasionally wart-like spots. Flowering occurs preferentially on canopy or opened area, beginning with the onset of the rains in austral summer (November) and can last up to April (pers. obs.). The inflorescences carry 40-50 flowers and the large canary-yellow flowers are characterized by a red-velvet lip (Portères 1954). The anther is composed by four yellow and limp pollinia separated by a rostellum from the stigma which is situated below and ventrally. The cylindrical fruits are straight or a little arched and measure in mean 18-20 cm but up to 25 cm (Portères 1954).

Reproductive biology

As insufficient numbers of accessible flowers were available in the forest reserve of Sohoa for reproductive biology experiments, other individuals from small remnant populations (Boungoudranavi, M'Bouzi, Moya, M'Tsamoudou and Saziley) (Figure 1) were also used.

Floral measurements

In November-December 2010, 15 flowers were randomly collected from 13 inflorescences and stored in 70% ethanol prior to measurements. Floral characters were measured to the nearest 0.01 mm using a digital caliper (Figure 2). A total of 41 inflorescences from 18 individuals located in Boungoudranavi (5), M'Bouzi (1), Moya (1), M'Tsamoudou (9) and Sohoa (2) were used to measure the mean total length of an inflorescence using a tape measure (in centimetre) and the mean number of flowers per inflorescence. The number of inflorescences per individual and the number of simultaneous opened flowers per individual were assessed on 19 individuals from Boungoudranavi (5), M'Bouzi (1), Moya (3), M'Tsamoudou (3) and Sohoa (7). Floral volatiles were analysed using the solid phase micro extraction (SPME) technique (Zhang and Pawliszyn 1993). Three exposures were realised *in situ* from three different individuals in Bougoundranavi (1) and M'Tsamoudou (2), and two analyses were realised *ex situ* on flowers from accession CR0108 (Comoros) from the Biological Resource Centre (BRC) Vatel in Reunion Island (Roux-Cuvelier and Grisoni 2010). Fibres were exposed to the flower headspace in a glass bell-jar for 3 to 8h30 between 10 am and 7 pm.

Pollinator observations

Pollinator observations were realized using two hard-disk camcorders (Sony DCR-SR90E and Sony DCR-SR72E) mounted on tripods with long-lasting batteries (NP-FP90, NP-NH100 InfoLithium® P and H series rechargeable battery), and protected with a waterproof casing (Sony SPK-HCB marine sport pack). Videotape sessions were conducted in November-December 2010 on three study sites of Grande Terre (Boungoudranavi, M'Tsamoudou and Sohoa) and one of Petite Terre (Moya). The target flower was examined for pollen removal and/or deposition before and after each videotape session.

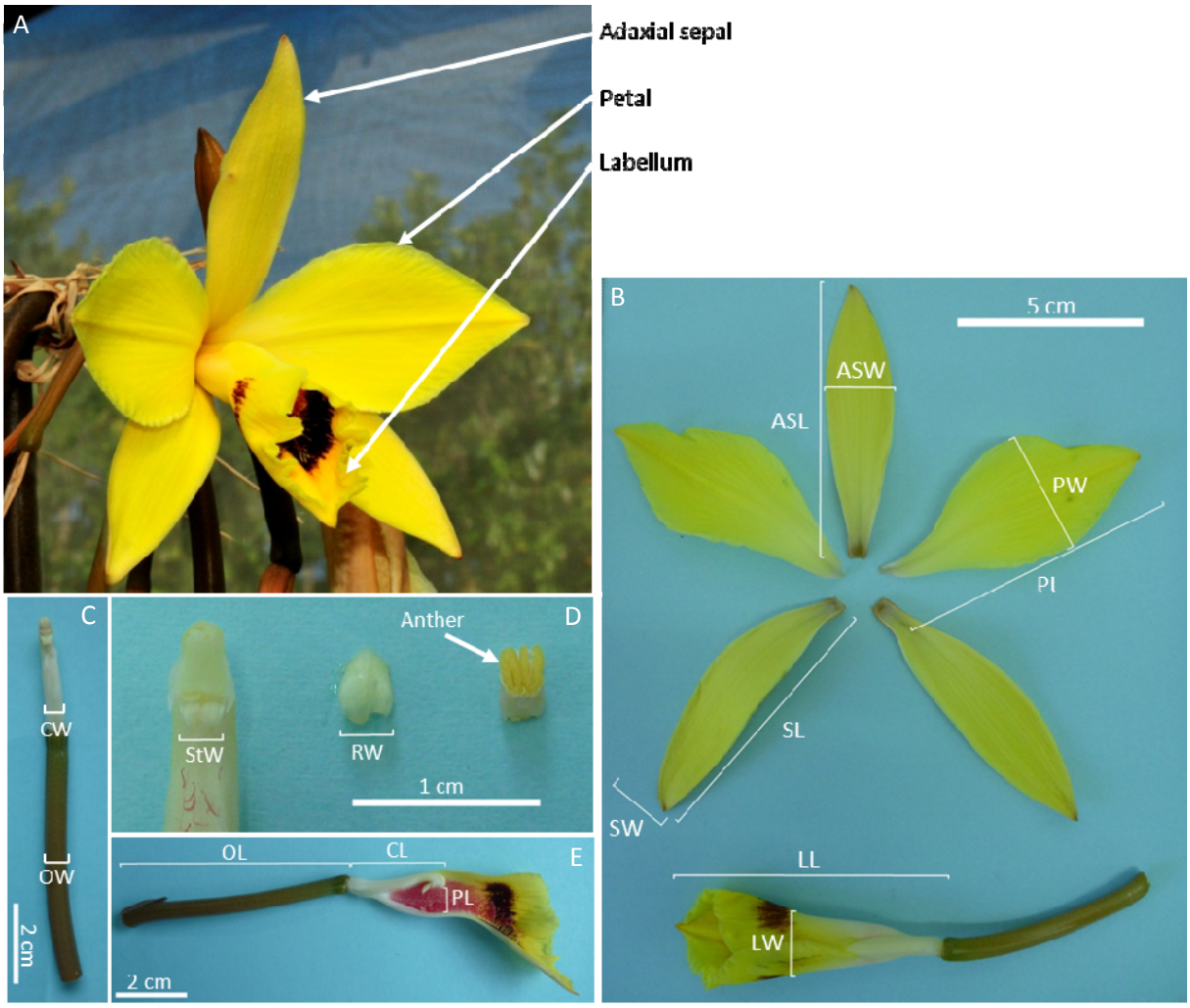

Figure 17 Figure 2 Floral characters measured on the flowers of *V. humblotii* (A); (B) Adaxial sepal length (ASL) and width (ASW); Petal length (PL) and width (PW); Sepal length (SL) and width (SW); Labellum length (LL) and width (LW); (C) Column width (CW) and ovary width (OW); (D) Stigma width (StW) and rostellum width (RW); (E) Ovary length (OL), column length (CL) and pollinator linked trait (PL). Photos R. Gigant

Natural fruit-set

The mean natural fruit set per flower was estimated using 74 inflorescences from 24 individuals from Boungoudranavi (6), M'Bouzi (1), Moya (3), M'Tsamoudou (9), Saziley (1) and Sohoa (4) by counting the number of fruits produced per flower per inflorescence (evaluated by the floral scars).

Breeding systems and compatibility of *Vanilla humblotii*

Breeding experiments were performed on 34 flowers from Boungoudranavi (11), Moya (5) and M'Tsamoudou (18). Spontaneous self-fertility was tested on 16 flowers from eight inflorescences (one inflorescence per individual) by the exclusion of insect visits using insect-proof bags. Auto-compatibility was tested by hand self-pollinations using eight virgin flowers from seven inflorescences (1-2 flowers per inflorescence with one inflorescence per individual). Nine cross-pollination treatments were performed as described in Nielsen (2000). Both male and female parents were carefully recorded and further microsatellite analyses (see below) permitted to differentiate six true cross-pollinations from three geitonogamous pollinations (involving different individuals with the same genotype ie clones) To avoid limitation of fructification due to resource allocation, only inflorescences without fruit were used and no more than three flowers per inflorescence were employed to receive pollinia. Inflorescences were protected by an insect-proof bag. Early abortions were recorded after 12-18 days after pollination experiments. Unsuccessful pollinations can be identified a few days after pollinations by the fall of the faded flower and the absence of swelling of the ovary (Lecomte 1901; Shadakshari et al. 2003). Comparisons between self- and cross-pollination were performed using Fischer exact test with the software R 2.12.2 (R Development Core Team 2010).

Genetic analyses

Sampling of *V. humblotii* in the Sohoa population

The patch-like distribution of the individuals is probably related to the vegetative propagation as described in many *Vanilla* species (as reviewed in (Bory et al. 2010; Gigant et al. 2011a)). Without *a priori* knowledge on the extent of the clonality in *V. humblotii,* our sampling was based on a transect covering the entire distribution of the individuals in Sohoa. Given the difficulty to distinguish between stems which have arisen vegetatively from those arisen from seedlings, an inter-sampling of 5 m was defined for individuals in continuity, and 1 m when they were on different supports. An irregular sampling with numerous orthogonal transects permitted to include a large range of

neighbour distances which is considered as more efficient for pattern detection than a regular lattice (Fortin et al. 1989). Following this method, 49 ramets were sampled in the population of Sohoa and their spatial coordinates were carefully recorded. Stem fragments were collected and stored in silica gel for further DNA analyses.

DNA isolation and genotyping

DNA extractions were made from the CTAB protocol (Risterucci et al. 2000) adapted for 1g of lyophilized stem for the leafless *Vanilla* species (pers. modifications). This is the first use on a large sampling of 10 microsatellite markers from *V. humblotii* and *V. roscheri* (Gigant et al. 2011b) and mVplCIR031 from *V. planifolia* (Bory et al. 2008b). The PCR conditions described in (Gigant et al. 2011b) were identical for all markers easing the multiplexing

Genetic diversity

To measure the extent of clonality in the population of Sohoa and its influence on the genetic structure, two datasets were created: a dataset 'ramets' composed by the entire set of sampled individuals and a dataset 'genets' composed by the different genotypes after clone exclusion (one individual was randomly selected for each clone). The clones were identified using the multilocus analysis of clonality available in GenAlex 6.41 (Peakall and Smouse 2006). Individuals with genotypes lacking at least one marker were excluded from this analysis. Using GenAlex, the probability (*Pse*) was calculated for each repeated multilocus to exclude the possibility that a specific repeated multilocus could be generated by sexual reproduction under random mating. The genotypic diversity, as assessed for clonal plants by G/N (Ellstrand and Roose 1987), was calculated as the number of 'genets' divided by the number of 'ramets'. The average maximal clonal patch size was estimated from the pairwise geographical distance option from GenAlex, between each ramet with the same genotype

Genetic variability was estimated for each microsatellite locus using GenAlex on the dataset 'genets' by the number of different alleles (N_a) and the number of effective alleles (N_e). Heterozygosity observed (H_O), heterozygosity expected under random mating (H_E) and fixation indices (F_{IS}) were

estimated using the software Genepop 4.1 (Rousset 2008). Deviations from Hardy-Weinberg equilibrium (HWE) were tested at marker and population levels by the exact P-values estimations of the Markov chain method proposed by Guo and Thompson (1992) implemented in Genepop (1000 dememorizations, 100 batches and 1000 iterations per batch). Evidence of null allele was estimated with Microchecker 2.2.3 (Van Oosterhout et al. 2004) and linkage disequilibrium over loci was tested using Fischer's exact tests of Genepop 4.1 and Bonferroni correction for multiple comparisons.

Fine-scale Spatial Genetic Structure

Fine-scale Spatial Genetic Structure (FSGS) analysis was performed for the Sohoa population for which reliable autocorrelation could be provided due to more than 30 pairwise comparisons in each distance class (Wartenberg, cited in Waser and Mitchell (1990)). The marker mVroCIR05 was excluded from the analysis due to a significant deviation from HWE. Autocorrelations analyses based on the multilocus pairwise kinship coefficients of Loiselle (Loiselle et al. 1995) were calculated using the software Spagedi 1.3 (Hardy and Vekemans 2002). The coefficient values were regressed on the linear and natural logarithm of the geographical distances between the individuals. The respective slopes of those two regressions were b_d and b_{Ld} and their corresponding coefficients of determination R_d^2 and R_{Ld}^2. Five individuals at the limit of the area of the distribution of the population were excluded from the analysis (> 500m from most individuals) to avoid stretching the distance classes. Eight distance classes were defined automatically by the software Spagedi 1.3, to equilibrate the number of pairwise comparisons between the distance classes. The average multilocus kinship coefficients were calculated for each distance class defined separately in each dataset. The distance classes calculated for the two datasets 'ramets' and 'genets' were similar with 0-10 m, 10-22 m, 22-32 m, 32-42 m, 42-53 m, 53-63 m, 63-75 m and 75-105 m for the dataset 'ramets' and 0-10 m, 10-23 m, 23-33 m, 33-43 m, 43-54 m, 54-63 m, 63-75 m, 75-105 m for the 'genets'. Standard errors for the multilocus estimates of the kinship coefficients per distance class were assessed by jackknifing data over the loci. The significance of the kinship coefficients and slope estimates (*b*) were tested by

comparing the observed values with those obtained after 10 000 random permutations of the individuals among positions.

The *Sp* statistic (Vekemans and Hardy 2004) was calculated to quantify the strength of the spatial genetic structure of the two datasets. It is defined by $Sp = b_{Ld} / (F_1 - 1)$, where F_1 is the mean kinship coefficient between pairs of neighbours in the first distance class (< 10 m).

2D LSA

Given the patchy distribution of the individuals, we further examined the spatial genetic structure of the individuals used in the FSGS analysis, by the heuristic two-dimensional local spatial autocorrelation analyses (2D LSA) (Double et al. 2005) as implemented in GenAlex. This method based on pairwise comparisons estimated the local autocorrelation between a pivotal point and its nearest neighbours. Permutation testing and a 1-tailed test at $P = 0.05$ was used. The 2D LSA was computed for a range of nearest neighbours from 1 to 25, but only the result with the maximum number of significant autocorrelations is shown.

Estimation of gene dispersal

An iterative approach implemented in Spagedi was used to estimate jointly the neighbourhood size (*Nb*) and the gene dispersal (σ_g) in the restricted distance range $\sigma_g > d_{ij} > 20\ \sigma_g$, knowing the density of individuals (*D*) in the population of Sohoa and with d_{ij} the geographical distance for *i-j* pairs of individuals. *D* was estimated for *V. humblotii* in Sohoa as 65.5 ind/ha as given by the number of genets sampled per hectare. Obviously, this value does not represent the density of *V. humblotii* in Mayotte (due to large agricultural landscapes) but it reflects the density of genets in the same fragment of primary forest. Neighbourhood size (*Nb*) was approximated from the global natural regression slope of kinship coefficients by $(F_1 - 1) / b_{Ld}$. As an approximation of D_e, the effective density determined from *D* (N_e / N), where N_e / N is the effective population size per the population size, *D* / 2, *D* / 4 and *D* / 10 were used because demographic studies have shown previously that N_e / N varied from 0.1 to 0.5 in adult plant populations. Gene dispersal (σ_g) was estimated from *Nb* and D_e using $\sigma_g = (Nb / 4\pi D_e)^{1/2}$. The procedure of estimation of the parameters by iterations failed to

converge when the regression of autocorrelations became null at one step, or when σ_g became larger than the distance between *i-j* pairs of individuals in the range $\sigma_g > d_{ij} > 20\ \sigma_g$.

The relative contributions of pollen (σ_p) and seed (σ_s) dispersals to the total gene flow were estimated on the dataset 'genet' following the method of (Heuertz 2003). A cubic regression was fitted on the average kinship coefficients per distance class and the logarithm of the distance. The shape of the regression can be described by the *k*-value, the second derivative of the term of second and third power of the cubic regression (for details see (Vekemans and Hardy 2004; Jacquemyn et al. 2006)). A concave shape (*k*-value > 0) signifies a leptokurtic gene flow implying a restricted seed dispersal compared to pollen dispersal ($\sigma_s << \sigma_p$) in the case of a convex shape (*k*-value < 0) $\sigma_s >> \sigma_p$.

3.2.4 Results

Reproductive Biology

Floral characteristics of *V. humblotii*

The mean length of an inflorescence was estimated at 37.5 (± 10.4) cm with 33.9 (± 12.4) flowers produced per inflorescence. An individual carried 5.3 (± 3.7) inflorescences on average. A mean number of 1.84 (± 2.1) simultaneously opened flowers on the same individual was estimated. Floral features of *V. humblotii* are reported in Table 1.

Tableau 9 - Table 1 Mean (± SE) of floral measurements in millimeters (N = 15).

Floral segments	Length	Width
Adaxial sepal	66.0 (± 1.2)	15.5 (± 4.7)
Lateral sepal	65.2 (± 2.1)	17.6 (± 4.5)
Petal	69.3 (± 3.7)	29.0 (± 1.5)
Lip	60.1 (± 0.8)	9.8 (± 1.5)
Column	24.8 (± 0.2)	3.5 (± 0.5)
Ovary	56.7 (± 6.5)	4.7 (± 0.4)
Rostellum	na	2.7 (±0.4)
Stigmata	na	1.8 (±0.4)

The petals were slightly longer and much wider (66%) than the sepals. Observations and measurements of the flowers fitted with the description of *V. humblotii* by Portères (1954). Little

variation of the floral traits was observed between flowers excluding the length of the ovary. The height between the lip and the pollinarium was measured at 5.5 (± 0.5) mm and the inside width at 7.1 (± 1.5) mm. No volatile components were detected after SPME analysis in any of the flowers tested.

Breeding systems and natural fruit set

None of the autonomous self-pollination tests led to fruit development suggesting a pollinator-dependent system to achieve sexual reproduction (Table 2). Self-compatibility of the species was verified and no significant differences were detected with the cross-pollination treatments (P = 1). Seventeen fruits were recorded on 2179 flowers evaluating the natural fruit set at 0.8 %.

Tableau 10 Table 2 Reproductive success obtained from the breeding experiments.

	No of flowers	Fruit set (%)
Pollinator excluded	16	0.0
Combined auto-pollination	12	83.3
Self-pollination	9	88.9
Geitonogamy	3	66.7
Cross-pollination	6	83.3

Pollination ecology

In November-December 2010*,* flowers were fully opened at around 9 am when the sunlight became significant and began to close in early afternoon to fade at sunset; therefore the lifespan of unpollinated flowers was estimated to be 1 day. These observations exclude night pollination events and supported our choice to realize the floral scent captures and video recordings during the day. Recordings (46h) spanned over 11 days of observation. Two potential pollinators were identified: a small female carpenter bee which was captured in Sohoa and further identified by Pr Connal Eardley (Plant Protection Research Institute, Pretoria, South Africa) as *Allodape obscuripennis* Strand (Apidae, Xylocopinae, Allodapini; Figure 3 a and b) and a female souimanga (*Nectarinia coquerelli ,* Passeriformes, Nectariniidae; Figure 3 c). No pollen movement was observed neither in the recorded flowers nor in any flower examined during field observations. A total of 23 visits of *A. obscuripennis*

was recorded with one visit every two hours in mean. The insect remained from 2 sec to 90 sec inside the flower with a mean visit duration of 29 sec. All the visits were concentrated on three days of recording and in only two sites (Boungoudranavi and Sohoa) which increased the rate at almost 4 visits / hour for a "visiting day". Although no pollen movement was observed, one recording permitted to film the arrival of an *A. obscuripennis* on a *V. humblotii* flower with pollinia on the mesonotum (upper surface of the mesothorax) (Figure 3 b). The single visit of a *V. humblotii* flower in Moya study site by the souimanga lasted for 38 s. The recording showed that perched on an inflorescence of *V. humblotii,* it was looking for food all around the flowers, in the insertion of the petals where sometimes small insects can be found. Otherwise, flowers of *V. humblotii* were also visited by small arthropods, ants, midges and predatory spiders which use the flower as attractive trap for small insects.

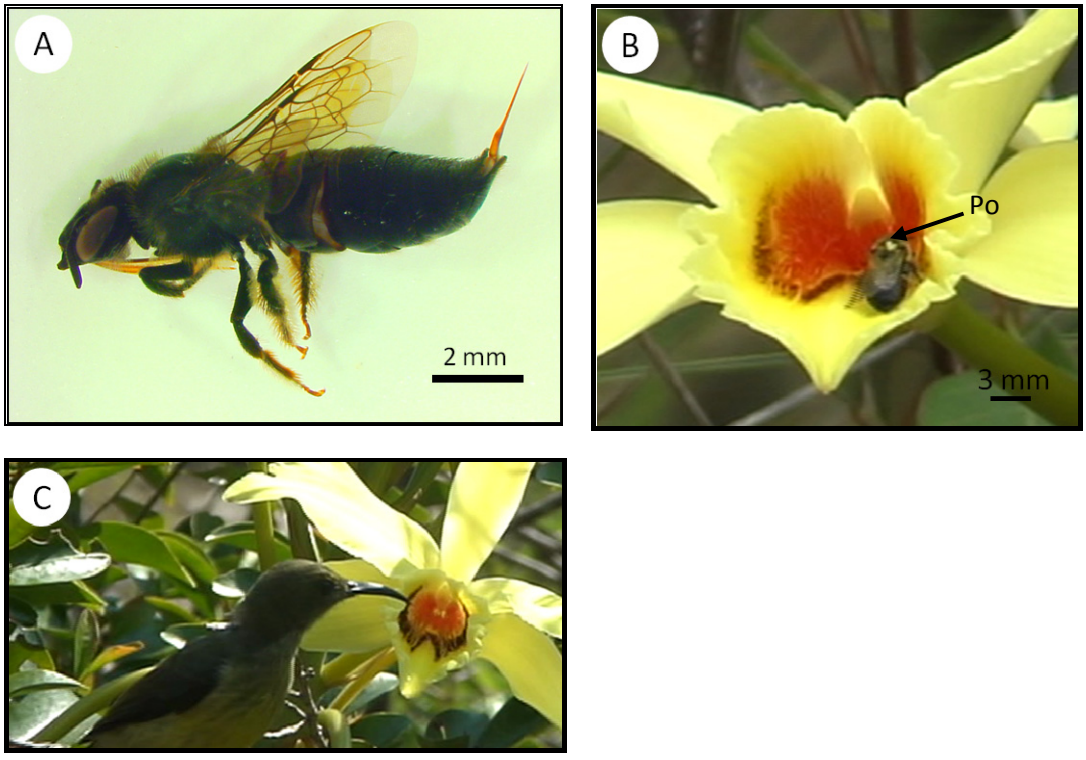

Figure 18 - Figure 3 Visitors of *V. humblotii* flowers: (A) *Allodape obscuripennis* sampled visiting a flower of *V. humblotii*; (B) *A. obscuripennis* landing on *V. humblotii* flowers with pollinia (Po) on the mesonotum; (C) Female *Nectarinia coquerelli* probably foraging insects on *V. humblotii* flowers. Photos (A) A. Franck; (B) and (C) R. Gigant.

Genetic analysis of Sohoa population

Genetic diversity

Among the 49 ramets of *V. humblotii* sampled in Sohoa population, one was excluded because genotypes lacked for at least one marker to identify the clones. A total of 42 genets were revealed, with a genotypic diversity (G/N) measured at 0.88 representing 12.5% of clonality (*Pse* < 0.001). Therefore all the repeated genotypes found in Sohoa were considered as derived from vegetative reproduction. Clones were represented by four repeated genotypes (Figure 4) and presented a phalanx distribution where clonal ramets are spatially aggregated (Lovett-Doust 1981). On the other hand, spatial distribution showed that neighbour individuals were not necessarily clones, which *a posteriori* justified our sampling strategy.

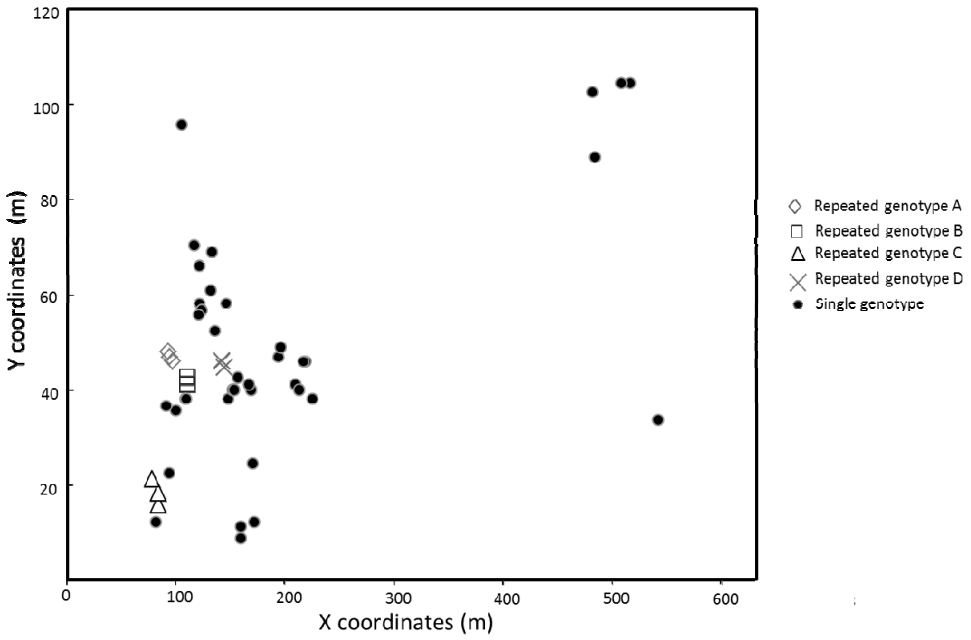

Figure 19 - Figure 4 Distribution of the ramets sampled in the population Sohoa with the positions of each clone identified after multilocus analyses available in GenAlex 6.41.

Genetic variability estimates for the 'genets' of the Sohoa population (42 individuals) are reported in Table 3. Because significant departure from HWE was detected for the marker mVroCIR05 following the exact P-values estimations, this marker was removed from the subsequent FSGS analysis. Neither

significant linkage disequilibrium between markers after Bonferroni correction nor significant levels of null alleles were detected in the study.

Tableau 11 - Table 3 Genetic variability per marker and across markers calculated for 11 polymorphic microsatellite loci in the *V. humblotii* Sohoa population. Number of different alleles (N_a), number of effective alleles (N_e), heterozygosity observed (H_O), heterozygosity expected under random mating (H_E) and fixation indices (F_{IS}) are precised. Global F_{IS} value for Sohoa population was calculated from the mean H_O and H_E values.

Locus	N_a	N_e	H_O	H_E	F_{IS}
mVplCIR031 *(EF486655)*	2	1.237	0.214	0.193	-0.108
mVhuCIR03 *(JN222562)*	4	1.913	0.381	0.484	0.213
mVhuCIR04 *(JN222563)*	2	1.690	0.429	0.413	-0.038
mVhuCIR06 (*JN222564*)	2	2.000	0.667	0.504	-0.323
mVhuCIR07 (*JN222565*)	2	1.825	0.405	0.458	0.117
mVhuCIR08 *(JN222566)*	2	1.024	0.024	0.024	0.000
mVhuCIR09 *(JN222567)*	5	2.633	0.548	0.629	0.129
mVroCIR09 *(JN222578)*	2	1.825	0.357	0.459	0.222
mVroCIR05 *(JN222575)*	3	2.201	0.452	0.553	0.183*
mVroCIR03 *(JN222573)*	6	2.621	0.500	0.628	0.203
mVroCIR04 *(JN222574)*	4	2.479	0.548	0.605	0.094
Mean Sohoa population (± SD)	3.09 (± 1.45)	1.950 (± 0.522)	0.411 (± 0.175)	0.450 (± 0.187)	0.086*

* indicates significant deviation from Hardy Weinberg equilibrium ($P < 0.05$).

FSGS of the Sohoa population

Comparing the spatial kinship coefficients for distance class one (F_1), the autocorrelation between individuals separated by less than 10 m for the 'ramets' is significant and more than 4-folds greater than for the 'genets' subset (Table 4; Figure 5). Consistently, the *Sp* statistic calculated for the 'ramets' was 2-folds higher than for the 'genets', which indicated a stronger FSGS for the 'ramets' than for the 'genets' (Table 4). Furthermore, the F_1 value for the 'ramets' is significant ($P < 0.01$) while not significant for the 'genets' ($P > 0.05$). These results suggested a strong influence of the repeated multilocus genotypes in the first distance class, with a spatial pattern appearing completely random after exclusion of the clones.

Tableau 12 - Table 4 Estimates of FSGS and gene dispersal parameters for the two datasets 'ramets' and 'genets': Loiselle's average kinship coefficient between individuals separated by less than 10 m (F_1) and its standard deviation (SD), slope of linear regression (b_d) and log-normal regression (b_{Ld}) and their respective correlation coefficient R_d^2 and R_{Ld}^2, intensity of SGS (*Sp*), Neighborhood size (N_b), gene dispersal distance (σ_g) and standard error in parenthesis for three effective densities (D_e) estimated from the density of individuals (D). Lack of value (-) indicates a failure of the estimation procedure. N_b is a value obtained from the mean estimate under the three assumed D_e with standard deviation (SD) in parenthesis.

Pop	F_1 (SD)	b_d (R_d^2)	b_{Ld} (R_{Ld}^2)	*Sp*	N_b (SD)	σ_g (m) ($D_e = D/2$)	σ_g (m) ($D_e = D/4$)	σ_g (m) ($D_e = D/10$)
Ramets	0.052**	-0.001***	-0.042***	0.045	21.6	25.2	33.0	44.1
	(0.015)	(0.031)	(0.038)		(4.1)	(7.8)	(3.4)	(-)
Genets	0.012	-0.000**	-0.019*	0.020	18.6	21.2	31.6	45.0
	(0.016)	(0.000)	(0.007)		(1.6)	(3.29)	(6.2)	(-)

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.05$

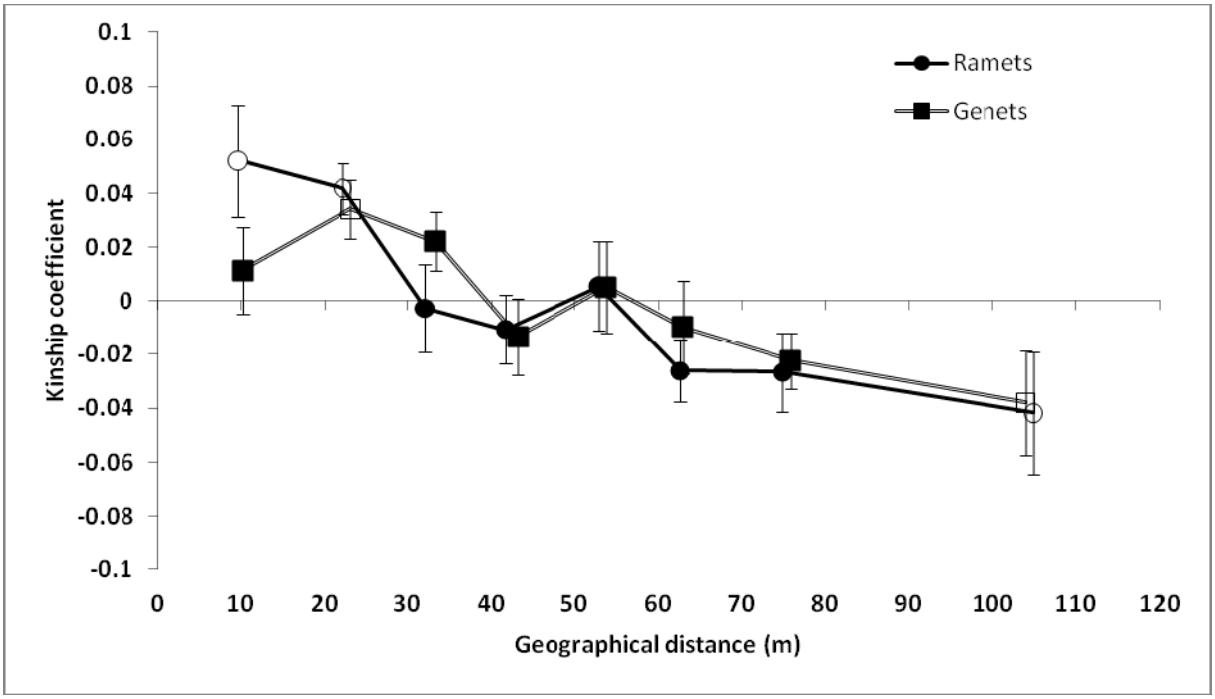

Figure 20 - Figure 5 Correlograms showing the spatial structure for the two datasets 'ramets' and 'genets' of the population of Sohoa, with average Loiselle kinship coefficients over all loci (±SD) plotted as a function of the geographical distance in meters. Significant values of kinship coefficients are precised by empty symbols (P (2-sided test) < 0.05).

The average maximal clonal patch size was measured at 4.6 (±2.7) m. The FSGS obtained in the population of Sohoa showed a significant linear relationship for the two datasets whatever the linear

regression or the natural logarithm regression, with a decrease of kinship coefficients correlated with an increase of geographical distance (Figure 5). A better fit was obtained between genetic and geographical with the log-linear transformation of geographical distance (R_{Ld}^2 (ramets) = 0.038 and R_{Ld}^2 (genets) = 0.007) than with the linear distance (R_d^2 (ramets) = 0.031 and R_d^2 (genets) ≈ 0.000) (Table 4). Furthermore, where the correlogram intercepts the *X*-axis or switches sign permitted to define the average patch size of autocorrelation which dimensions are determined by breeding structure and dispersal (Epperson and Clegg 1986), and influenced by density (Antonovics and Levin 1980). The average autocorrelation patch size was estimated graphically at around 50 m for the two datasets (Figure 5).

2D LSA

The results of 2D LSA showed local positive genetic autocorrelations for 19 nearest neighbours between 'genets' (genetically more similar than average) in the population of Sohoa, therefore exclusively due to sexual reproduction. The graphical representation highlighted three patches of autocorrelations composed not only by nearest neighbours but also by more distant individuals (Figure 6).

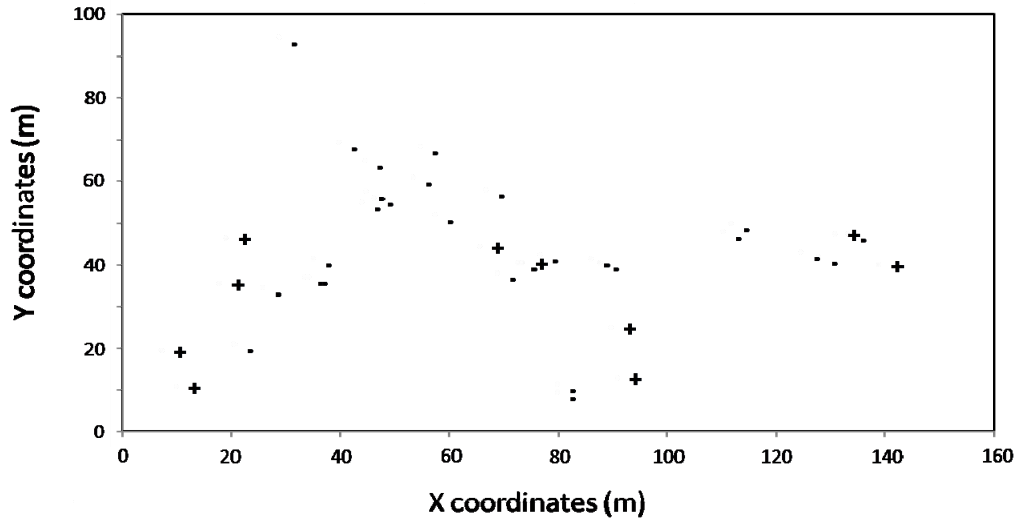

Figure 21 - Figure 6 Spatial distributions of the significant autocorrelations (+) detected in the dataset 'genets' using 2D LSA analysis ($P < 0.05$).

Gene dispersal

Dispersal parameters converged for the two ratios *D* / 2 and *D* / 4 (as approximations of D_e) but the procedure failed to estimate the parameters based on *D* / 10 (Table 4). Neighbourhood size estimates were higher for the 'ramets' than for the 'genets' (Table 4). Gene dispersal estimates were in the same range of variation regardless of the datasets but slightly greater at small distance for the 'ramets' than for the 'genets' (Table 4).

The shape of the regression of the kinship coefficients and the logarithm of the distance for the dataset 'genets' was convex (*k*-values > 0) indicating a pollen dispersal more restricted than seed dispersal ($\sigma_p << \sigma_s$).

3.2.5 Discussion

Vegetative reproduction importance and impact on the spatial genetic structure in Sohoa

For plants with both sexual and asexual reproduction, the vegetative reproduction impacts on the spatial genetic structure of asexual and sexual regeneration of populations (Chung and Epperson 1999; Hossaert-McKey et al. 1996; Shapcott 1995). At fine scale, the spatial genetic structure is expected to be higher for the 'ramets' than the 'genets' in case of significant structuring effect of clonal growth (Heywood 1991). In the Sohoa population, 12.5% of the individuals were clones deriving from vegetative reproduction (Figure 4). This value is an agreement with the proportion of clones (6-25%) detected in two other leafless *Vanilla* species, *V. claviculata* and *V. barbellata,* studied in Puerto Rico using allozymes (Nielsen 2000). Vegetative reproduction by natural stem cuttings of the vine is probably the predominant reproduction mode for most *Vanilla* species, as described in *V. planifolia, V. bahiana*, *V. chamissonis*, *V. madagascariensis*, *V. dilloniana*, *V. barbellata*, *V. claviculata* (reviewed in (Bory et al. 2010; Gigant et al. 2011a)).

The impact of clonality was further analysed in *V. humblotii* by a FSGS analysis. The main difference observed between the two datasets occurs in the first distance class for which, after exclusion of the repeated multilocus genotypes, the autocorrelation became not significant (Table 4). In the dataset

'ramets', individuals separated by short distances (< 10 m) are more genetically related than those that are further apart, due to the patch-like distribution of the clonality (Figure 4). Obviously, the vegetative reproduction increases greatly the genetic structure of the population as evidenced by *Sp* statistics (Table 4). The phalanx distribution of the clonal architecture (Lovett-Doust 1981) (Figure 4) is mainly responsible for this prominent structuring in the first distance class (Vallejo-Marín et al. 2010).

Clonal propagation also plays an important role in gene dispersal. Alberto et al. (2005) in the dioecious seagrass *Cymodocea nodosa* revealed that vegetative reproduction can represent a gene flow component as important as sexual reproduction. In Sohoa, the gene dispersal is somewhat influenced by vegetative reproduction as witnessed by σ_g values that tend to be slightly higher for the 'ramets' than for the 'genets' (Table 4). This differential effect observed between the two species is compatible with the guerilla clone strategy and low genotypic diversity (< 0.5) in *C. nodosa* (Alberto et al. 2005) as opposed to a phalanx strategy and high genotypic diversity (0.88) in *V. humblotii*.

Sexual reproduction in *V. humblotii*

As for most *Vanilla* species (Bory et al. 2008c; Gigant et al. 2011a), *V. humblotii* exhibited two reproduction modes: sexual and asexual. Hand-crossing experiments revealed that *V. humblotii* is auto-compatible (Table 2). Spontaneous self-pollination is however prevented by the presence of a large rostellum covering the entire stigmatic surface (Table 1), therefore the species depends on pollinators for sexual reproduction. The *Sp* estimates (*Sp* (genets) = 0.020, Table 4) are concordant with the range of variation found in species with animal-dispersed pollen (mean 0.017 ± 0.0142 as reviewed in Vekemans and Hardy (2004)). The average F_{IS} value measured at 0.086 witnessed the mixed mating system with a predominant allogamy. Allogamous sexual reproduction in *V. humblotii* was confirmed by the relatively high genotypic diversity revealed (H_E = 0.450, Table 3).

Pollinator dependent system

Apart from in America, there is a considerable lack of information about potential *Vanilla* pollinators and this is, to our knowledge, the first report of pollinator visits for African *Vanilla* species. The small

female carpenter bee *A. obscuripennis* and the sunbird *N. coquerelli* were clearly identified as visitors and we will further discuss why they might be potential pollinators of *V. humblotii* in Mayotte.

In the absence of euglossine bees in Africa (Van der Cingel 2001) and given that allodapine bees (family Apidae, Xylocopinae, Allodapini) have an old world distribution and are most diverse and abundant in subsaharan Africa (Michener 1975; Schwarz et al. 2003; Chenoweth 2011), they might be *Vanilla* pollinators for African *Vanilla* species. The relatively small neighbourhood size measured at 18.6-21.6 from the genetic autocorrelation analyses is consistent with a bee-pollinated plant (Chung and Epperson 1999). Allodapine bees are on average 7.0 mm long (C. D. Eardley in Ley and Claßen-Bockhoff (2009)) and up to 3-5 mm high according to our observations. Given that we have measured the space between the anthers and the base of the lip at 5.5 (± 0.5) mm, the size of the bee may be enough to make contact with the anthers when penetrating to the bottom of the flower given the long and numerous hairs (1-3 mm) lining the labellum. We indeed observed one allodapine bee with pollinia on the mesonotum (Figure 3B). Usually, yellow flowers are known to attract Anthophoridae insects as are red and white flowers (Robertson 1927). Given the absence of nectar and flower scent in *V. humblotii*, pollination is rewardless but on the other hand, female allodapine bees can collect pollen for nest feeding so acting as pollen thieves (Hargreaves et al. 2010), and in this particular case, the pollen of *V. humblotii* could be considered as a reward. Recently, in South-Africa two other small carpenter bees (*Allodapula* sp.) were identified as potential pollinators of the rewardless flowers of *Vanilla roscheri,* (Gigant et al. in prep). Some *Diuris* and *Thelymitria* Australian rewardless species of Orchidaceae are also pollinated by allodapine bees (Van der Cingel 2001). The females of *Allodape sp* were invoked to pollinate nectariferous *Gladiolus brevifolius* (Iridaceae) in South Africa (Goldblatt et al. 1997) and the flower of *Welwitschia mirabilis* in Namibia (Welwitschiaceae) (Wetschnig and Depisch 1999). Other events of pollination with *Allodape sp* were identified in the crops *Mangifera indica* in South Africa (Eardley and Mansell 1993) and *Daucus carotta* in India ((Kumar et al. 1989) as cited in (Sharma 2004)) but the bees were not characterized as the main pollinator.

A female sunbird *N. coquerelli* was also observed visiting a flower of *V. humblotii* in the study site Moya. Called locally "souimanga", it is mainly nectarivorous and well known to pollinate *Aloe mayottensis* (Pailler et al. 2002) but its diet is also composed by invertebrates (Louette 1988). Bird pollination is often associated with large tubular scentless red or yellow flowers (Hingston and Quillan 2000), floral characters acting as stimuli to attract birds, but the flowers have usually large quantities of low concentrated nectar (Nicolson 2002). In the absence of nectar reward in *V. humblotii*, the small insects in the flowers (midges, spiders and ants) could also induce bird visits since it is suggested in this foraging behaviour when its beak penetrates the tube, it may remove pollen from the anther or load pollen from the stigma. In Australia, Timewell and Mac Nally (2004) suggested that meliphagid honeyheaters could consume insects attracted to flowers. In some orchid species, ants have been shown in some cases to be attractors to bird pollinators (Knox et al. (1985) as cited in Peakall (1994)). Birds were already identified as visitors and potential pollinators of *Vanilla* species. Hummingbirds in Oaxaca (Mexico) (Lubinsky and Seung-Chul 2006) were observed visiting the flowers of *V. planifolia* but without pollen removal and more broadly, they were long considered as vanilla pollinators in Tropical America (Bouriquet 1954; Stéhlé 1954) although this was never experimentally shown. Similarly, rare natural pollination events of the introduced *V. planifolia* were supposedly attributed to noticed visits of an endemic *Zosterops* (Zosteropidae) in Reunion island (Bory et al. 2008c) and of a sunbird from the *Cynniris* genus in Madagascar (Bouriquet 1954).

Interestingly, the pollination of the same species by allodapine bees and sunbird was already described in *Aloe maculata* (Aloeaceae) in South Africa (Hargreaves et al. 2010). Furthermore, the two pollinators are often known to be active in dry environment (Aloeaceae (Nicolson 2009; Hargreaves et al. 2010), Welwitschiaceae (Wetschnig and Depisch 1999), Aizoaceae (Peter et al. 2004)), therefore it would not be surprising to find them as *V. humblotii* pollinators although this will need to be confirmed by pollen movement observations.

Natural fruit-set

Whatever the pollinators of *V. humblotii,* they show low abundance or effectiveness given the absence of pollen movement observed and the low natural fruit set recorded (0.8 %). In *V. humblotii*, like in many rewardless orchids, fructification is rather pollinator than resource limited (Tremblay et al. 2005), as demonstrated by the high success of our hand-pollination experiments. Leafless *Vanilla* species in Puerto Rico exhibited a much higher fruit set: from 14.5% (*V. dilloniana*) to 18.5% (*V. barbellata*) (Tremblay et al. 2005) as in South Africa for *V. roscheri* (26.3% (Gigant et al. in prep). In Mayotte, pollinators availability might be impacted by an hostile surrounding degraded landscape which might prevent plant-pollinator interaction (Aguilar et al. 2006; Andrieu et al. 2009). On the other hand, our results are compatible with low levels of fruit set (≤ 1%) already reported in America for *V. planifolia*, *V. pompona* and *V. riberoi* (as reviewed in (Gigant et al. 2011a). Globally, these values are in agreement for the general low fruit set reported in orchid species, of which over 70% have natural fruit set below 10%, with significantly lower values described for deceptive than rewarding species (Tremblay et al. 2005). Furthermore, we observed predation of flower buds (> 20%) in numerous sites probably caused by introduced rats, at least in M'Bouzi where there is no freshwater (data not shown) which might also severely impact the reproductive success.

Impact of the sexual reproduction on the genetic structure of Sohoa population

Without vegetative reproduction, the spatial genetic structure of the population depends mainly on pollen transfer and subsequent seed dispersal and recruitment. The 2D LSA analysis illustrated the pattern of autocorrelations between nearest neighbours related to sexual reproduction (the dataset 'genets') in the population of Sohoa (Figure 6). Three main patches of autocorrelations can be identified comprising not only nearest neighbours but also distant ones and some nearest neighbours in each patch are not systematically related. These results highlight the non-significant autocorrelations obtained at the shortest distance (< 10 m) for the 'genets' following FSGS analysis based on Loiselle coefficients (Figure 5), with lower *Sp* value (0.020) detected for the 'genets' than for the 'ramets' dataset (0.045) (Table 4). Given that we detected a more important seed flow than

pollen flow in Sohoa, we hypothesize that pollen flow is probably restricted between near neighbours in the population (due to bee-pollination), and that a mechanism of seed dispersal is present and is responsible for the absence of significant structuring in the shortest distance class (ie no seedlings beneath the mother plant).

Possible seed dispersal mechanisms

Hamrick and Trapnell (2011) have described the ability of spatial genetic structure analyses to provide insights into patterns of within population seed dispersal. More structuring is expected for herbaceous plants than trees with similar mechanisms of pollination and seed dispersal (Hamrick and Loveless (1989) cited in Chung and Epperson (1999)), this being explained by the height of the plant. For numerous terrestrial orchid species with flowers close to the ground, a leptokurtic distribution of seed dispersal (falling down beneath the mother plant) is detected (Machon et al. 2003). On the other hand, for epiphytic orchids, given the height of canopy and the dust-like seeds character of most orchids (Dressler 1981), dispersal by wind or water movements on hillsides will involve a subsequent recruitments of individuals not necessarily close to the mother plant. In *Vanilla* species, most of the blooming and fruiting occur preferentially on canopy, up to 8 m high for *V. humblotii* in Mayotte depending on the vegetation. In most *Vanilla* species, seeds are associated with a moist pulp (Madison 1981), but this is not the case for all species. In *V. humblotii*, this character is mainly verified in the first days after dehiscence since the fluid ultimately dries up releasing the seeds (pers. obs.). Householder et al. (2010) observed also in *V. bicolor*, the non-oily character of the fruits and suggested a mixture of wind and gravity for seed dispersal. This could also be the case for *V. humblotii* and could explain the general pattern of isolation-by-distance revealed associated with an absence of spatial genetic structuring in the first distance class for the 'genets' dataset.

In America, the moist pulp of fragrant *Vanilla* fruits was described as favouring epizoochory by insects or vertebrates. Euglossine bees were indeed demonstrated as seed dispersers as they are attracted by fragrant fruit and collect scent for mating (Householder et al. 2010; Van Dam and Householder 2010). Stingless bees (*Trigona sp*) were also observed carrying *Vanilla* seeds although

without scent collecting behaviour (Householder et al. 2010). Evidence of bat dispersal was also reported for *V. pompona* (N. Byrd, as cited in Soto Arenas and Dressler (2010)) and suggested in *V. insignis* (Soto Arenas and Dressler 2010). Occasional herbivory was detected for *V. pompona* subs. g*randiflora* in Peru (Householder et al. 2010) and was supposedly attributed to large vertebrates such as bats or marsupials, although herbivory seems to be extremely rare (Householder et al. 2010). *V. humblotii* fruits are not scented (pers. obs.) but given the early moist character of the seeds following dehiscence, a possible epizoochorous seed dispersal cannot be ruled out as we observed, once, fruit herbivory in M'Tsamoudou population (data not shown). In Mayotte, there are three species of bats, *Chaerephon leucogaster* and *C. pusillus* and the large frugivorous *Pteropus seychellensis comorensis* well known to disperse seeds of native plants in the western Indian Ocean islands (O'Brien 2011).

Consequences of reproduction mode of *V. humblotii* on the genetic diversity in Sohoa

Despite a physical barrier mechanism (rostellum) promoting outcrossing by a pollinator-dependent system, geitonogamous inbreeding might occur in the *V. humblotii* Sohoa population as we demonstrated that *V. humblotii* is auto-compatible, as are most orchids (Tremblay et al. 2005). An inflorescence of *V. humblotii* produces one flower per day, sometimes two, but an individual carried 5.3 (± 3.7) inflorescences on average with a mean number of 1.84 (± 2.1) simultaneous flowers per plant, making geitonogamous matings likely (Eckert 2000) and this is reinforced by the limited pollen transfer between nearest neighbours in a bee-pollinated species. Furthermore, geitonogamy probability will be strongly reinforced by the phalanx architecture of clonality (clumped distribution) increasing the opportunity of matings between clones (Charpentier 2002; Reusch 2001). In Sohoa, significant but weak signs of inbreeding were detected (F_{IS} = 0.086), and only one marker was concerned (mVroCIR05). Due to restriction of pollen dispersal, consanguineous mating may occur and would result in a Wahlund effect responsible for the weak heterozygote deficit observed (Wahlund 1928). Also this could be attributed to slight genetic drift or local selection. Clonal diversity was already described as inversely correlated with selfing rate (Eckert 2000; Albert et al. 2008).

Clonal diversity is high in Sohoa (0.88) compared to what is commonly found in other clonal species (mean genotypic diversity = 0.42 (Vallejo-Marín et al. 2010)). In Puerto Rico, leafless *Vanilla* species showed similar clonal diversity to *V. humblotii* and all populations were at Hardy Weinberg equilibrium (Nielsen and Siegismund 1999). The high clonal diversity therefore probably reduces probability of geitonogamy. Moreover, to limit deleterious consequences of extensive geitonogamous mating, natural selection should promote characters increasing the clonal diversity and the establishment of a high number of genotypes within a patch (Albert et al. 2008). Scentless and rewardless flowers, as found in *V. humblotii*, are such characters limiting pollinator visits between close inflorescences, driven by pollinator learning behaviour (Internicola et al. 2006), in accordance with the geitonogamy-avoidance hypothesis (Dressler 1981; Jersakova and Johnson 2006; Jersáková et al. 2006; Johnson and Nilsson 1999; Johnson et al. 2004; Smithson and Gigord 2001). By these mechanisms, species experiencing a deceptive pollination system are supposed to limit the genetic consequences of inbreeding at the scale of a population (Johnson et al. 2004). Interestingly a genetic study of wild populations of *V. planifolia* from Mexico (Soto Arenas 1999b) revealed extremely high levels of inbreeding ($F_{IS} \approx 1$), which might be associated both with the patchy distribution of the individuals (a single clone that may cover an area up to 0.2 ha) and their low density (less than one plant found per square kilometer) (Soto Arenas 1999b) and with the fact that *V. planifolia* flowers are scented and contain 1-8-cineol, a strong attractant for euglossine bees (Soto Arenas & Dressler 2010). This demonstrates that some *Vanilla* species may experience strong negative effects of geitonogamy. On the contrary, in *V. humblotii*, the combination of a high clonal diversity and rewardless flowers considerably limit the deleterious inbreeding effects expected from geitonogamous mating which should be expected due to clonality and auto-compatibility.

3.2.6 Conclusion

For the first time, this study provides precious knowledge on the reproductive biology and its influence on the genetic diversity and the spatial structuring in an African *Vanilla* species. As most

Vanilla species, *V. humblotii* exhibited a mixed mating system with a deceptive-system to perform allogamous sexual reproduction and vegetative reproduction due to natural stem cuttings (Tremblay et al. 2005; Gigant et al. 2011a; Bory et al. 2008c; Bory et al. 2008d). Whether the allodapine bee *A. obscuripennis* and the souimanga *N. coquerelli* are pollinators of *V. humblotii* will need to be confirmed by further observations providing evidence for pollen movements. But this will be difficult given that pollen movements seem uncommon in Mayotte, and that the average natural fruit set measured is low (0.8 %). Using microsatellite markers, the genetic analysis permitted to detect FSGS in accordance with a pattern of isolation-by-distance (Epperson and Allard 1989; Wright 1943). The phalanx architecture of the clonality is likely responsible for the positive autocorrelations in the shortest distance class but its harmful effects on the genetic diversity (inbreeding) are limited by a high genotypic diversity and a deceptive pollination limiting geitonogamy.

More field observations have to be made to confirm our hypotheses regarding pollen and seed dispersal in *Vanilla humblotii,* but these results and particularly those from the genetic analysis represent a crucial step towards the definition of a conservation plan for this species in Mayotte. For this purpose, further field sampling will have to be made at a larger scale over Mayotte covering all extant populations, to thoroughly study the impact of fragmentation on the genetic diversity to propose adapted conservation strategies.

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3.3 Article 3

Impact of fragmentation on the genetic diversity of *Vanilla humblotii* Rchb. f. (Orchidaceae) in Mayotte (Comoros Archipelago, Indian Ocean) and implications for conservation

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3.3.1 Abstract

Background Found in the Comoros Archipelago, *V. humblotii* Rchb. f. (Orchidaceae) is indentured to semi-xerophytic forests that have considerably suffered from an intense deforestation. In Mayotte, where the species is officially protected, the populations are scattered throughout the territory and population size vary considerably with many stations being reduced to few individuals.

Methods Genetic diversity and its spatial structuring in nine remnant populations of *V. humblotii* in Mayotte were assessed using 11 microsatellite loci.

Results Levels of heterozygosity were relatively high (H_E = 0.331) even in small populations, while allelic richness was correlated with sample size and high frequency of private allele (> 0.1) were revealed. All populations, but one, were at Hardy Weinberg equilibrium. Most of the genetic diversity was found within populations, associated with a mean moderate genetic differentiation (F_{ST} = 0.115) varying among populations. Isolation-by-distance pattern (< 10 km) and geographical barriers were mainly responsible for the clustering/isolation of the geographical populations with some exceptions of long distance dispersal events detected.

Conclusions Recent bottleneck and long-lived clonal ability have limited the short term negative effects of habitat fragmentation on genetic diversity, but preliminary signs of deterioration of the population genetic dynamics are revealed by the loss of allelic richness for small populations and a slight heterozygous deficiency in one population. The short term threat is mainly anthropogenic, but the long term survival of *V. humblotii* appears limited in the absence of signs of sexual reproduction. Conservation guidelines should consider the fragmented diversity at the scale of Mayotte, probably by appealing to *ex situ* conservation methods, and focus the *in situ* conservation on the last large populations still able to multiply vegetatively and sexually.

Keywords: genetic diversity, clonality, fragmentation, *Vanilla humblotii*, microsatellite, orchids.

3.3.2 Introduction

Fragmentation of the habitat modifies the structure of a metapopulation by the isolation of individuals in small populations which can impact severely the variability of the populations by the loss of genetic diversity (Aguilar et al. 2008; Allendorf et al. 2002; Endels et al. 2007; Frankel 1974; Frankham 1995; Hughes et al. 2008; Laikre et al. 2010). Nevertheless, plant populations do not respond the same way to habitat fragmentation (McGarigal and Cushman 2002; Young et al. 1996) although a size reduction of populations is often followed by loss of allelic diversity (Allphin et al. 1998), reduction of heterozygosity (Alemayehu et al. 2011; Allphin et al. 1998) following genetic drift, inbreeding and the reduction of interpopulation gene flow (Young et al. 1996; Templeton et al. 1990). Consequences on genetic diversity are complex, depending on the reproduction biology of the species (Hamrick et al. 1993; Wright 1943; Kalisz et al. 2001), the dependence on pollinator to perform sexual reproduction (Ashworth et al. 2004; Harris and Johnson 2004; Steffan-Dewenter and Tscharntke 1999), and on the mechanisms of seed dispersal (Hamrick et al. 1993; Wright 1943; Kalisz et al. 2001; Aguilar et al. 2006). Aguilar (2008) revealed that the losses of alleles and polymorphic loci can be more pronounced for outcrossing species than non-outcrossing and selfing species, because the majority of genetic diversity is found at the population level. Therefore, due to the loss of genetic diversity, metapopulations can be exposed to reduction in fitness (González-Astorga and Núñez-Farfán 2001) and local extinction (Ellstrand and Elam 1993), and disturbances of this kind could affect not only the long-term survival of the populations but also the evolutionary potential of the species to respond to environmental changes (Kéry et al. 2000).

In Mayotte (Comoros Archipelago), climax forest has disappeared in flat lowlands since the nineteenth century after an intense deforestation for sugarcane, cocoa and coffee plantations (Guéneau 2006; Pascal et al. 2001). Quantitative data reporting changes in the forest cover from 1947 show massive losses which stopped between 1969-1987 (reforestation period) (DAF 2002). Since 1987, about 6000 ha of woodlands have again disappeared in Mayotte (DAF 2002) and

nowadays, relictual natural forests that have survived agricultural and forest exploitation are mainly on hilltops. These persisting forests concentrate the majority of the archipelago's floral richness on a very tiny proportion (15 km²) of the whole land area of Mayotte (374 km²) (Vos 2004; Pascal et al. 2001).

Vanilla Plum. ex Miller genus (Vanilloideae sub-family, Vanilleae tribe and Vanillinae sub-tribe) is a monophyletic primitive lineage of the Orchidaceae family (Cameron 2004, 2005) containing around 110 species distributed throughout the tropics but absent from Australia (Portères 1954). In most cases, the species are hemi-epiphytic vines growing in tropical wet forests between 0-1000m (Portères 1954). However, adaptation to xeric conditions by leaflessness (18 species) appeared at least three times during the evolution of the genus (Bouetard et al. 2010). One clade of seven leafless species is found in the Southwest Indian Ocean (SWIO) area : Madagascar, Seychelles, Comoros, Zanzibar, Pemba and the east African coastline from Tanzania to South Africa (Portères 1954). *Vanilla humblotii* Rchb. F. is indentured to semi-xerophytic forests in the Comoros archipelago (Mayotte, Moheli, Anjouan, Grande Comore) (Cribb and Hermans 2009; Lecoufle and Bosser 2011), possibly also present in the north of Madagascar (Cribb and Hermans 2009). The species is officially protected in Mayotte since 2006 (Arrêté Préfectoral APn° 42/DAF/2006 3rd may 2006) together with 110 other plant taxa identified by the Conservatoire Botanique National de Mascarin (CBNM) (Barthelat et al. 2006). Data regarding the status of the species in the Comoros are lacking but a recent survey in Anjouan suggested that the species might be on the edge of extinction (L Gigord, com. pers.). In Mayotte, the species is threatened by habitat fragmentation due to anthropogenic pressure.

We recently demonstrated two reproduction modes in *V. humblotii*: vegetative by natural stem cuttings, and sexual. Although *V. humblotii* is self-compatible, outcrossing is promoted by a large rostellum which prevents passive contact between pollinia and stigmata (Gigant et al. in prep). Possible pollination by an allodapine bee (*Allodape obscuripennis*) and a souimanga (*Nectarinia coquerelli*) were suggested in Mayotte (Gigant et al. in prep). Outcrossing behaviour and dependence

on pollinator could therefore make this species highly vulnerable to fragmentation. Knowledge on the genetic diversity and population genetic structure is an essential prerequisite to conservation management of endangered plant species. We therefore used a set of previously developed microsatellite markers (Gigant et al. 2011b; Bory et al. 2008a) to address the following questions: a) What is the level of genetic diversity retained in *V. humblotii* remnant populations in Mayotte? b) How is the genetic variation distributed across populations and what is the differentiation pattern? The questioning will be viewed through the prism of the impacts of the mixed reproduction mode on the genetic and spatial structuring of the populations. These results are essential to understand the genetic consequences of demographic decline and fragmentation of remnant *V. humblotii* populations so that adequate *in situ* or *ex situ* conservation measures could be taken.

3.3.3 Materials and methods

Study site

Mayotte (15° 33’ S, 54° 31’ E) is a French Overseas Territory belonging to the Comoros Archipelago situated between Madagascar and Mozambique. The drift of the Somali plate on top of a hot spot plume is at the origin of the emergence of the archipelago (Emerick and Duncan 1982; Späth A. et al. 1996). Mayotte is composed by two islands: Grande-Terre and Petite-Terre (Figure 1) and culminates at the Benara Peak (660 m). The past volcanic activity has shaped the topography of Mayotte formed by old lava flows and elongated hills (Audru et al. 2010).

Ecological description of the study sites

Although most of the remnant populations of *V. humblotii* were found in dry forest (and dry thickets) (Boungoudranavi, Chiconi, Handrema, M’Bouzi Moya, M’Tsamoudou, Saziley) for which the species is particularly well adapted, this orchid was also present in a mesophylous forest (Sohoa) and on the slopes of Choungui Mount at medium altitude (Figure 1).

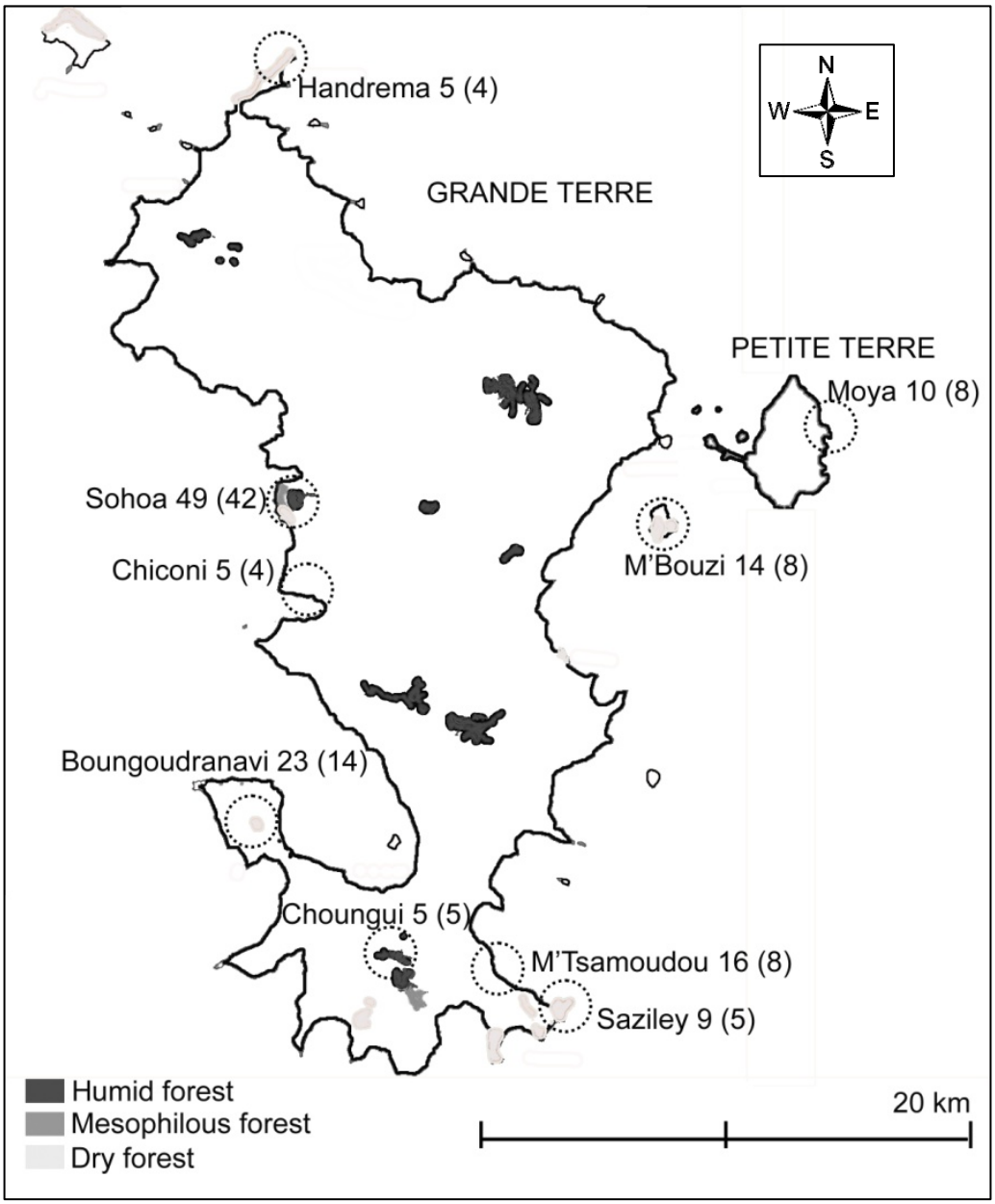

Figure 22 - Figure 1 Map of Mayotte showing the sampling sites of *V. humblotii.* The nine remnant geographical populations are precised with the number of ramets sampled and the number of genets identified after multilocus genotype analysis in parenthesis. Remnant natural vegetation relicts are shown.

V. humblotii with other species is considered as a diagnostic species of the dry forest (Rolland and Boullet 2005) which covers nowadays 361 ha composed of 90% native trees and shrubs (Laybourne 2010; Pascal et al. 2001). *Mimusops comorensis* Engl. (Sapotaceae), *Commiphora arafy* H. Perrier (Burseraceae), *Poupartia gummifera* Sprague (Anacardiaceae) form a sparsely tree stratum of 10 m

height (Rolland and Boullet 2005; Pascal et al. 2001). Of similar species richness but different in aspect, the dry thicket results from the extreme xeric conditions preventing forest formation and reducing the trees to shrubs of 5 m high (Pascal et al. 2001). This vegetation is disseminated on the coast and has likely composed most of the forests in the islands around Grande Terre, where there are still relics in M'Bouzi and M'Tsamboro (Pascal et al. 2001). In terms of abundance, the invasive species such as *Lantana camara* L. (Verbenaceae) and *Furcraea foetida* L. (Agavaceae) predominate the environment preventing any species to thrive (Pascal et al. 2001). The mesophylous forest covers currently only 85 ha but it was likely much more extended before the intense lowland clearings of the nineteenth century for agriculture (sugarcane, coffee and cocoa) (Pascal et al. 2001; Guéneau 2006). The population of Sohoa was described in details in Gigant et al. (in prep). Beyond 500 m of altitude, the plant formation of Choungui Mount is singular, predominated by *Monoporus bipinnatus* (Baker) Mez (Primulaceae) which is found nowhere else in Mayotte and associated with other rare species such as *Buxus madagascarica* Baill. (Buxaceae), *Cassipourea ovata* TuL (Rhizophoraceae), *Danais humblotii* Homolle (Rubiaceae) and where a new species was recently described *Ivodea choungiensis* Labat, Pignal & Pascal (Rutaceae) (Pascal et al. 2001).

Species description

Vanilla humblotii is a hemi-epiphytic leafless vine with inflorescences characterized by one or up to two ephemeral flowers opened per day (Gigant et al. in prep). The flowers are canary-yellow and characterized by a red-velvet lip (Portères 1954). Natural fruit set measured in 2010 was low (0.8 %) in Mayotte (Gigant et al. in prep).

The species is described locally abundant by the CBNM. It is probably due to the patch-like distribution of the individuals in relation with the vegetative propagation of the vines. For most *Vanilla* species, vegetative reproduction by natural stem cuttings of the vines (Bory et al. 2008b; Bory et al. 2008c; Gigant et al. 2011a) causes localised clonality at the population level (Gigant et al. in prep). In collaboration with the CBNM in Mayotte, the individuals of the nine remnant populations

were sampled and geo-localized during two field trips in August 2009 and December 2010 (Figure 1). The term population is applied here to a small and geographically discrete assemblage of ramets. Twenty centimetres of stem were collected per individual for DNA extraction. The entire distribution of the individuals in a population was covered to extract the maximum genetic diversity. The individuals of *V. humblotii* could be found in continuity on different supports, therefore it was often difficult to distinguish between stems deriving from vegetative reproduction from those arisen from seedlings. We sampled all separated individuals (with at least one meter between them) and every 5 m when the individuals were in continuity on different supports. Following this strategy, 5 to 49 ramets were sampled depending on the population, which represented a total of 136 ramets across Mayotte (Figure 1). The populations composed by only 5 ramets were sampled in their entirety (Chiconi, Choungui and Handrema). The largest population was Sohoa (49 ramets) for which the topography permitted to realize numerous orthogonal transects and it has been previously surveyed in details (Gigant et al. in prep).

Seven populations are situated in the biggest island, Grande-Terre: Handrema, Sohoa, Choungui, Chiconi, Saziley, Boungoudranavi and M'Tsamoudou (Figure 1). The two populations Moya and M'Bouzi are island populations sampled in Petite-Terre and M'Bouzi, respectively (Figure 1).

Two supplementary individuals of *V. humblotii* from Grande Comore (CR0108 and CR0870) available in the Reunion Biological Resource Centre (BRC) Vatel (Roux-Cuvelier and Grisoni 2010) were integrated in some genetic analyses.

Genetic diversity

Eleven microsatellite markers previously developed (Gigant et al. 2011b; Bory et al. 2008a) were used to assess the genetic diversity of the populations at the scale of Mayotte. DNA extractions and PCR conditions were described previously (Gigant et al. 2011b).

Due to the presence of clonality, two datasets were generated, one including all the sampled individuals ('ramets') and another ('genets'), after exclusion of identical multilocus genotypes

(clones). The clones were identified using the multilocus analysis of clonality available in GenAlex 6.41 (Peakall and Smouse 2006). The analysis required the exclusion of one individual from Sohoa and one from Boungoudranavi because genotypes lacked at least one marker. For each clonal genotype, the metric distance between the most distant individuals (maximum clone patch size) was determined using GenAlex. The probability (*Pse*) of a second encounter of a specific multilocus genotype generated by sexual reproduction under random mating was calculated for each multilocus genotype. This probability permitted to identify all clones derived from vegetative reproduction. Genotypic diversity as assessed for clonal plants was measured by G/N (number of 'genets'/number of 'ramets') (Ellstrand and Roose 1987).

The parameters of genetic variability and the tests of deviations from Hardy Weinberg equilibrium (HWE) were realized on the dataset 'genets'. Number of alleles per locus (N_a), number of effective alleles per locus (N_e) and Shannon's Information Index (I) were estimated using GenAlex for each polymorphic locus across populations. Observed heterozygosity (H_O), heterozygosity expected under random mating (H_E) and fixation indices (F_{IS}) were calculated with the Genepop 4.1 software (Rousset 2008) and the exact P-values estimations (Guo and Thompson 1992), implemented in Genepop were determined both at the marker level and at the population level to test deviations from random mating of HWE. Evidence of null allele was tested with Microchecker 2.2.3 (Van Oosterhout et al. 2004) and evidence of linkage disequilibrium between loci was tested using Fischer's exact tests available in Genepop with Bonferroni correction.

Factorial correspondence analysis

Using Genetix 4.05.2 (Belkhir et al. 2004), a multidimensional factorial correspondence analysis (FCA) was performed to identify clusters of individuals with related multilocus genotypes.

Differentiation of the populations

Population differentiation was further analysed by pairwise F_{ST} calculated as short-term distances between populations using Arlequin v 3.1 (Excoffier et al. 2005). The statistic procedure used 1000

permutations of haplotypes between populations and the *P*-value obtained was the proportion of permutations larger or equal to the observed pairwise F_{ST} value. A Bonferroni correction was applied on each *P*-value of the pairwise comparisons. Mean F_{ST} value averaged over all loci was also determined using Arlequin. Pairwise F_{ST} were calculated both for the 'ramets' and 'genets' datasets.

Mean number of migrants (*N*m) was obtained from the private allele method developed by Barton and Slatkin (1986), as implemented after correction for size in the software Genepop. This value represented the average number of migrants exchanged per generation according to an island model under neutrality and negligible mutation (Slatkin 1993). We also calculated the mean number of migrants obtained from pairwise estimates of *N*m as calculated in Arlequin based on Wright's F statistics.

Amova

Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed on the dataset 'genets' using GenAlex to determine the relative partitioning of genetic variation within and among populations.

Genetic structure

Genetic structure of *V. humblotii* populations were studied following two inferring approaches based on the dataset 'genets'.

Firstly, the software Geneland 3.3.0 (Guillot et al. 2005b) based on a Bayesian clustering incorporating the geographical coordinates of the individuals was used to infer the spatial discontinuities in the genetic data. The two individuals from Grande Comore were excluded from the analysis due to their geographical distance from the populations of Mayotte (around 200 km). The two-step approach recommended by Guillot (2005a) was used, by first inferring the number of clusters at the equilibrium (*K*) and second, holding (*K*) to verify assignations of the individuals to populations. Geneland analysis begun by running 10 replicates with a maximum rate of Poisson process fixed to 98 (the sample size), no uncertainty on the spatial coordinates, variation of *K* from 1

to 15 genetic clusters, maximum number of nuclei in the Poisson-Voronoi tessellation fixed to 294 (three times the sample size), 500 000 MCMC iterations with a burn-in period of 100 000, and a Dirichlet model in which allele frequencies are correlated. Once *K* was identified (second step), the MCMC was run 50 times with *K* = 9, with the same parameters as above. The mean logarithm of posterior probability was calculated for the 50 runs. The results of the top ten mean values were compared to verify the consistency of the results. Then, a spatial domain of 250 pixels along the X-axis and 400 pixels along the Y-axis permitted to check the runs visually for consistency.

Secondly, the model-based clustering method implemented in the program Structure 2.3.3 (Pritchard et al. 2000) was used for inferring the structure of the populations of *V. humblotii* in Mayotte also integrating the two individuals from Grande Comore. A Monte Carlo Markov chain (MCMC) algorithm is used to assess for the presence of an underlying genetic structure. From 1 to 15 genetic clusters (K) were *a priori* estimated for the calculation. For each, 5 runs were repeated with a burn-in period of 100 000 and 1 000 000 MCMC replications. A no-admixture model with correlated allele frequencies and location information was used to study our fully discrete populations (Hubisz et al. 2009; Pritchard et al. 2000). To provide a better estimate of the true number of genetic groups K, we calculated the modal value ΔK proposed by Evanno et al. (2005), an *ad hoc* statistic related to the change of the log probability of data with respect to K. Admixture proportions of individuals were visualized using Distruct v. 1.1 (Rosenberg 2004) with *K* = 7.

Isolation at large scale

Using the software Spagedi 1.3 (Hardy and Vekemans 2002), isolation-by-distance (IBD) at large scale was tested by analyses of autocorrelations based on the multilocus pairwise kinship coefficients of Loiselle (1995) calculated separately between all ‘genets’ and all ‘ramets’ sampled in Mayotte. The analysis took into account each multilocus genotype and their geographical position. The coefficient values were regressed on the spatial linear and natural logarithm of the geographical distance between the individuals. A number of 10 distance classes were empirically defined. The sizes of the

distance classes were estimated automatically by the software to homogenize the number of pairwise comparisons. Standard errors of the mean kinship coefficients per distance class were assessed by jackknifying data over the loci. The significance of the kinship coefficients and slope estimates were tested by comparing the observed values with those obtained after 10 000 random permutations of the individuals among positions.

3.3.4 Results

The number of 'ramets' and 'genets' are precised for each geographical population in Figure 1. The hypothesis that repeated multilocus genotypes could have appeared by sexual reproduction was excluded for each clonal genotype (*Pse* < 0.001, data not shown). Therefore, all the repeated genotypes were considered as true clones derived from vegetative reproduction.

Genetic diversity by locus

Parameters of genetic diversity obtained for the dataset 'genets' from Mayotte (98 individuals) are presented in Table 1. No significant departure from HWE was detected at the marker level following the exact P-values estimations of the Markov chain method. Neither significant linkage disequilibrium between markers after Bonferroni correction nor significant levels of null alleles were detected across the loci.

Genetic diversity by population

At the scale of Mayotte, the average genotypic diversity (G/N) was high (0.73) (27% clonality) (Figure 1, Table 2). It ranged from a minimum 0.50 (M'Tsamoudou) to a maximum of 1.00 in the Choungui population showing no repeated genotype (Table 2) and the values were not significantly correlated with fixations indices ($r^2 \approx 0.00$, $P = 0.99$). No repeated genotype was shared across multiple geographical populations. Maximum clone patch sizes varied greatly, from 1 m to 84 m with an average maximal size of 19.3 m (± 6.3). In most cases, the spatial clonal architecture was clumped with close aggregation of the clones (phalanx form) (Lovett-Doust 1981) (data not shown). But

extremely extended clones could have an intermingled distribution with other genotypes (Charpentier 2002), as detected in M'Tsamoudou for which the nine distant ramets (maximum clone size 84 m) had an interspersed distribution (data not shown).

Tableau 13 - Table 1 Comparison of the genetic variability per marker, calculated for 11 polymorphic microsatellite loci in the dataset 'genets' of *V. humblotii* populations. Number of different alleles (N_a), number of effective alleles (N_e), Shannon's Information Index *(I)*, heterozygosity observed (H_O), heterozygosity expected under random mating (H_E) and fixation indices (F_{IS}) are precised.

Locus	N_a	N_e	I	H_O	H_E	F_{IS}
mVplCIR031 *(EF486655)*	3	1.132	0.162	0.153	0.137	-0.118
mVhuCIR03 *(JN222562)*	5	1.419	0.432	0.367	0.372	0.012
mVhuCIR04 *(JN222563)*	2	1.367	0.345	0.347	0.323	-0.074
mVhuCIR06 (JN222564)	2	1.560	0.456	0.500	0.428	-0.169
mVhuCIR07 (JN222565)	3	1.787	0.644	0.459	0.467	0.017
mVhuCIR08 *(JN222566)*	2	1.127	0.119	0.102	0.078	-0.305
mVhuCIR09 *(JN222567)*	8	2.250	0.868	0.571	0.578	0.011
mVroCIR09 *(JN222578)*	4	1.501	0.431	0.286	0.366	0.220
mVroCIR05 *(JN222575)*	3	1.362	0.306	0.276	0.327	0.158
mVroCIR03 *(JN222573)*	10	2.215	0.883	0.520	0.581	0.104
mVroCIR04 *(JN222574)*	4	2.200	0.881	0.592	0.575	-0.029

Mean number of alleles per locus (*A*) was significantly correlated with sample size ($r^2 = 0.77$, $P < 0.01$) with values ranging from 1.636 (Handrema) to 3.091 (Sohoa) (Table 2). No significant correlation was obtained between population size and observed heterozygosity (H_O) ($r^2 = 0.09$, $P = 0.44$). No significant correlation was detected between F_{IS} and population size ($r^2 = 0.14$, $P = 0.32$). Significant deviation from HWE ($P < 0.05$) was only detected in the population of Sohoa associated with a significant deficit of heterozygote (Global Score U test for heterozygote deficit, $P < 0.001$) revealed by a slight positive F_{IS} value ($F_{IS} = 0.086$) as previously published (Gigant et al. in prep). A total of 13 private alleles were detected (Table 2). Only 3 of the private alleles (in the Sohoa population) were rare alleles (frequency < 5%).

The two accessions from Grande Comore (CR0108 and CR0870) showed important genotypic differences with Mayotte accessions (data not shown), providing together five additional private alleles distributed over four markers (mVhuCIR03, mVroCIR09, mVroCIR05, mVroCIR03).

Tableau 14 - Table 2 Genetic variability estimates from populations of *V. humblotii* with the number of ramets (No. ramet) and the number of genets (No. genet) precised; the following estimations are calculated based on the genet dataset: Clonal genotypic diversity (G/N), mean number of alleles per locus (*A*); percentage of polymorphic loci (P%); number of private alleles (Priv. alleles); observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}). Values in italic are precised for small populations (N ≤ 5).

Population ID	G/N	*A*	P%	Priv. alleles [Ŧ]	H_O	H_E	F_{IS}
Boungoudranavi	0.61	2.545	81.82	3 (2)	0.416	0.370	-0.122
Chiconi	*0.80*	1.818	63.64	*1* (1)	*0.409*	*0.288*	*-0.421*
Choungui	*1.00*	2.091	72.73	*0*	*0.436*	*0.389*	*-0.123*
Handrema	*0.80*	1.636	45.45	*1 (1)*	*0.159*	*0.224*	*0.288*
M'Bouzi	0.57	2.273	81.82	2 (1)	0.375	0.399	0.059
Moya	0.80	1.818	63.64	0	0.227	0.214	-0.065
M'Tsamoudou	0.50	2.273	100	2 (1)	0.432	0.387	-0.115
Saziley	*0.56*	1.727	63.64	*1* (1)	*0.273*	*0.259*	*-0.053*
Sohoa[1]	0.88	3.091	100	3 (0)	0.411	0.450	0.086*
Mean	0.72	2.141	74.75	1.86	0.349	0.331	-0.052

[Ŧ] Number of private alleles per population with in parenthesis the number of private alleles with frequency ≥ 10%

[1] Data from Gigant et al (in prep)

* Significant departure from Hardy Weinberg Equilibrium

Factorial correspondence analysis

The FCA illustrated the structure of the geographical populations based on the multilocus genotypes. The two first axes explained 40% of the total variation (Figure 2). The individuals of Boungoudranavi, Chiconi, M'Tsamoudou, and Sohoa were well individualised, owing to the contribution of their private alleles (data not shown) (Figure 2). The populations with fewer private alleles (Table2), Choungui, Moya, Handrema and Saziley were less differentiated in the analysis.

Genetic differentiation of the populations

The AMOVA partitioned 80% of the total genetic variation within populations (Table 3). Significant pairwise differentiations estimated by F_{ST} values were revealed for 38.9% of the pairs of populations ('genets') (Table 4). The significant F_{ST} values ranged from a low level of differentiation between Sohoa and M'Tsamoudou (F_{ST} = 0.067), to high levels between Moya and Choungui (F_{ST} = 0.233). Averaged over all loci, mean F_{ST} indicated a moderate differentiation between populations at the scale of Mayotte (F_{ST} = 0.115). When calculated from the 'ramets', the mean F_{ST} value increased (F_{ST} =

0.162) with 67% of significant pairwise F_{ST} values calculated between populations after Bonferroni correction (data not shown).

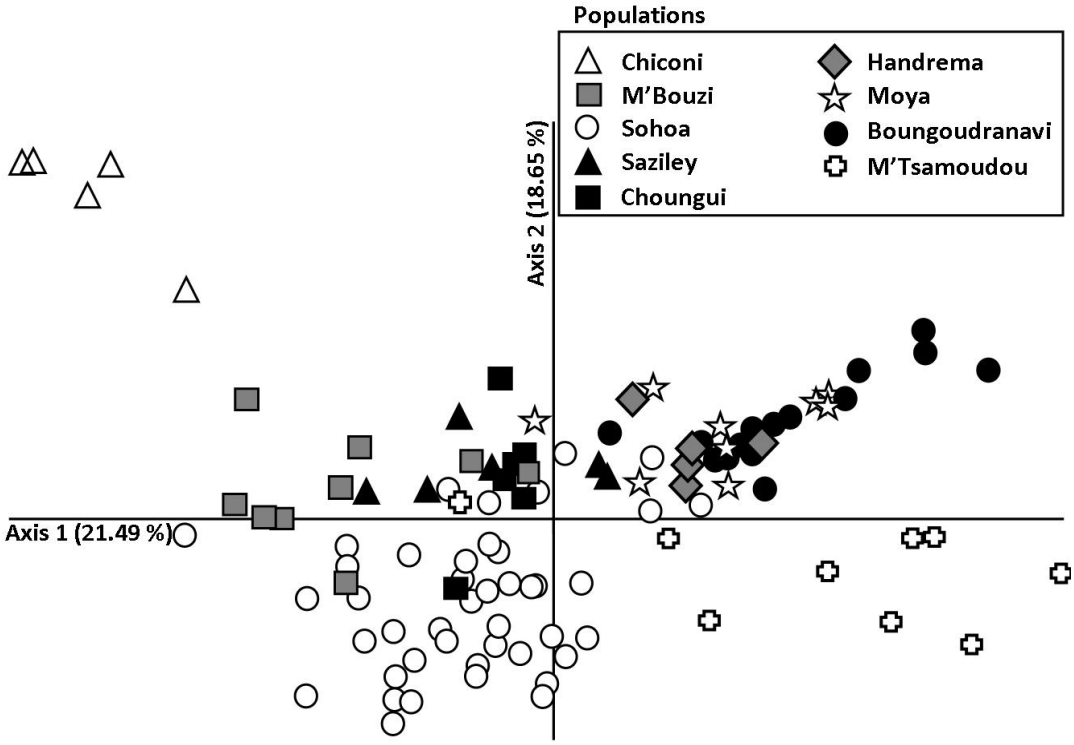

Figure 23 - Figure 2 2D-Factorial analysis of correspondences on the multilocus genotypes of the nine remnant populations of *V. humblotii* from Mayotte.

Considering only the populations with N > 5, pairwise F_{ST} measures showed that the Petite-Terre island population Moya was significantly differentiated at a moderate level ($0.128 \leq F_{ST} \leq 0.171$) from *V. humblotii* populations of Grande-Terre (Table 4).

Tableau 15 - Table 3 Analysis of molecular variance (AMOVA).

Source of variation	df	Variance component	Percentage of variation	Probability
Among populations	8	1.102	20	0.001
Within populations	89	4.291	80	
Total	97	5.393		

Tableau 16 - Table 4 Pairwise F_{ST} values are shown with in bold, the significant values obtained after Bonferroni correction. For easy viewing the populations with large effectives (N > 5) are grouped in the upper left part of the table. The values obtained with at least one population of the pair with N ≤ 5 are in italics. Mtsam = M'Tsamoudou ; Boung = Boungoudranavi ; Mbouz = M'Bouzi ; Handr = Handrema; Choun = Choungui ; Chico = Chiconi ; Sazil = Saziley.

	Moya	Mtsam	Boung	Mbouz	*Handr*	*Choun*	*Chico*	*Sazil*
Sohoa	**0.128**	**0.067**	**0.086**	0.037	***0.159***	*0.057*	***0.141***	*0.076*
Moya	-	**0.171**	**0.141**	**0.155**	*0.13*	***0.233***	*0.395*	***0.307***
Mtsam		-	**0.114**	0.088	*0.197*	***0.11***	*0.238*	*0.162*
Boung			-	**0.131**	*0.158*	*0.056*	***0.225***	***0.138***
Mbouz				-	*0.209*	*0.125*	*0.095*	*0.097*
Handr					-	*0.231*	*0.427*	*0.347*
Choun						-	*0.194*	*0.107*
Chico							-	*0.192*

The F_{ST} values also permitted to isolate significantly the population of Boungoudranavi ($0.086 \leq F_{ST} \leq 0.141$) from all the other populations of Mayotte. The island population M'Bouzi showed no genetic differentiation from the populations of Grande-Terre, apart from Boungoudranavi cited above. Finally, the differentiation between the two populations M'Tsamoudou and Sohoa from Grande-Terre was moderate but significant (F_{ST} = 0.067) and corresponded to a geographical separation of about 18 km (Figure 1; Table 4).

The mean number of migrants estimated from the pairwise F_{ST} values between populations was evaluated at an intermediate level (Nm = 1.790). As a complementary approach, the private alleles method used to estimate the number of migrants indicated a much lower number of migrants (Nm = 0.575).

Genetic structure

The spatial explicit approach of Geneland gave a mode at K = 9 from the posterior density distribution. The spatial explicit approach of Geneland gave a mode at K = 9 from the posterior density distribution (histogram not shown). The 10 best runs with K fixed to 9 showed consistent

genetic breaks in relation with the geographical location of the genetic clusters in Mayotte. However, a cluster predominantly composed by the geographical population of Saziley (with high posterior probability $p \geq 0.8$) comprised also three individuals of the population of M'Bouzi (less well assigned $p \geq 0.6$), distant of 20 km (Figure 3A), while there is an independent cluster for the geographical population of M'Bouzi gathering all the other individuals with high posterior probabilities ($p \geq 0.8$, Figure 3B).

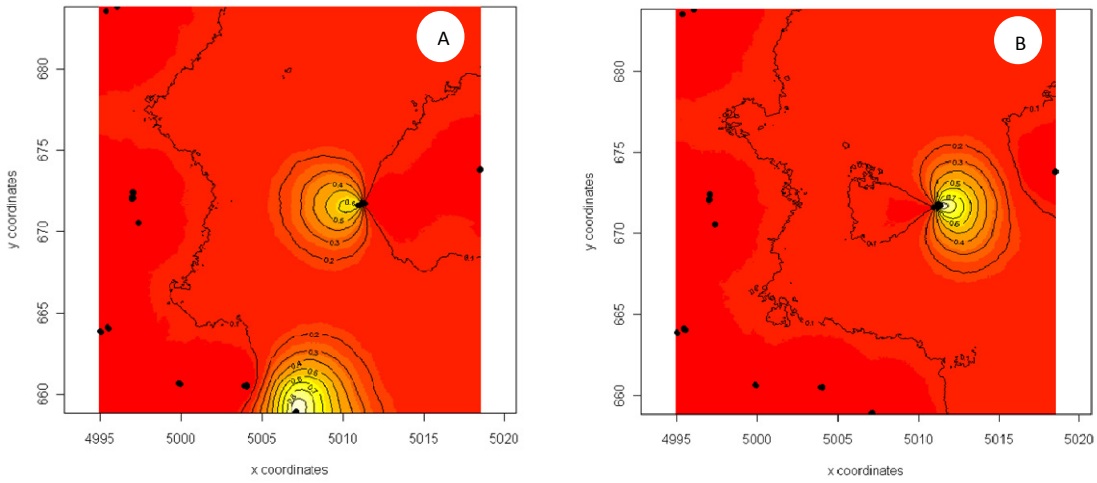

Figure 24 - Figure 3 Maps of the posterior probabilities (p) from the Geneland analysis. Each dark spot represents a discrete assemblage of plants. The cluster composed by the geographical population of Saziley and three individuals of M'Bouzi (A) and the independent cluster of M'Bouzi (B) are presented. The range of colors extends from white to red and corresponds respectively to high ($p \geq 0.8$) or low levels ($p \leq 0.2$) of posterior probabilities. Coordinates are given on each axis in UTM (km).

The ΔK value relying on the Structure results (Evanno et al. 2005) showed a break in the slope of likelihood values at $K = 7$ ($\Delta K = 7.19$) (Figure 4). The two individuals from Grande Comore were assigned to cluster 6 (Figure 4). Therefore the genetic structuring suggested at least six clusters at the scale of Mayotte for the nine remnant *V. humblotii* geographical populations. A strong assignment (posterior probabilities > 0.8) of members of a geographical population to a particular cluster was revealed for Sohoa (cluster 2), M'Tsamoudou (cluster 3), Boungoudranavi (cluster 4) and part of M'Bouzi population (cluster 5) (Figure 5). Cluster 1 grouped Handrema and Moya populations and cluster 7 Saziley and Chiconi (Figure 5).

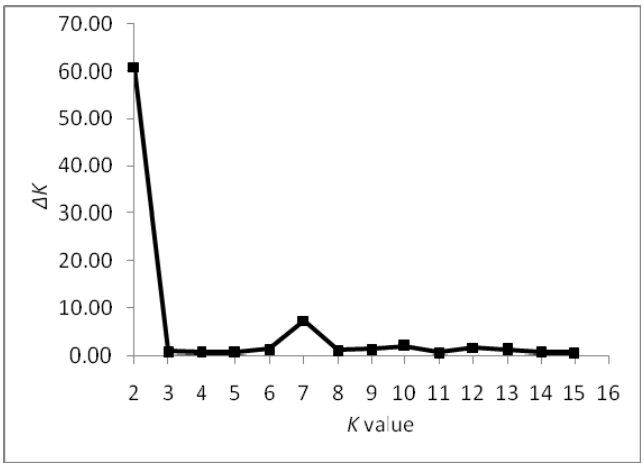

Figure 25 - Figure 4 Identification of the most probable number of cluster by *ΔK* (Evanno *et al.* 2005) obtained from Structure with *K values* ranging from 2 to 15. Each value was obtained by averaging the posterior probabilities of five independent runs.

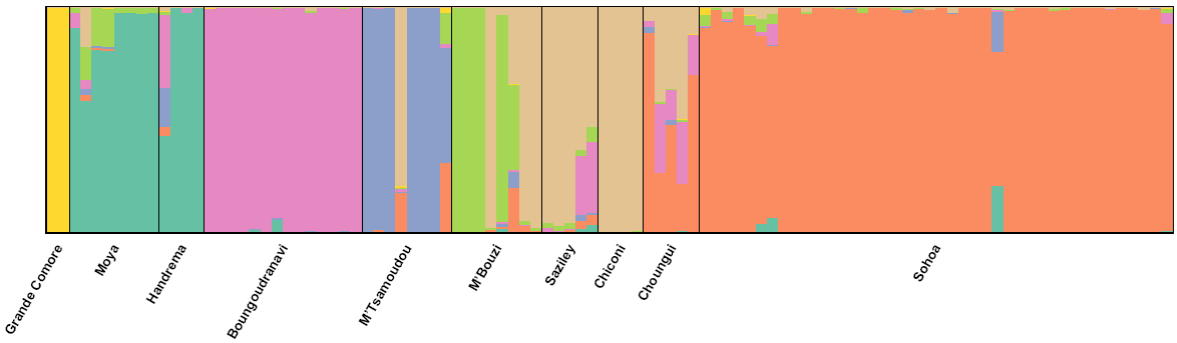

Figure 26 - Figure 5 Admixture proportions of the nine geographical populations sampled in Mayotte and two individuals from Grande Comore from the Structure analysis. The results are based on the multilocus genotype analyses of 11 microsatellite loci for the seven clusters identified (*K* = 7).

Individuals wrongly assigned to their own cluster (migrants) were detected: 3 individuals from M'Bouzi ($p > 0.9$) and 1 individual from M'Tsamoudou ($p = 0.795$) were clearly better assigned to cluster 7 (Figure 5). Twelve individuals less clearly assigned to a specific cluster represented admixture between multiple populations: These were 1 individual from each population Handrema, Sohoa, Moya, M'Tsamoudou, M'Bouzi ; 2 individuals in Saziley and all 5 individuals of the geographical population Choungui which had an assignment shared between clusters 2, 4 and 7 (Figure 5).

Isolation-by-distance at large scale.

The large scale spatial autocorrelations between individuals were similar for the two datasets with significant statistical regressions ($P < 0.001$). A better fit between genetic and geographical distances was obtained with the log-linear transformation of geographical distance (R_{Ld}^2 (ramets) = 0.088 and R_{Ld}^2 (genets) = 0.012) than with the linear distance (R_d^2 (ramets) = 0.035 and (genets) = 0.015). The mean autocorrelation analysis visualized by the correlogram (Figure 6) showed higher values with the dataset ('ramets'). For the two datasets, the autocorrelations showed a monotonous decreasing up to 10 km (log value = 9.16) and became erratic after this distance. The results were in accordance with the isolation-by-distance model expecting a decrease of autocorrelation coefficients correlated with an increase of the geographical distances.

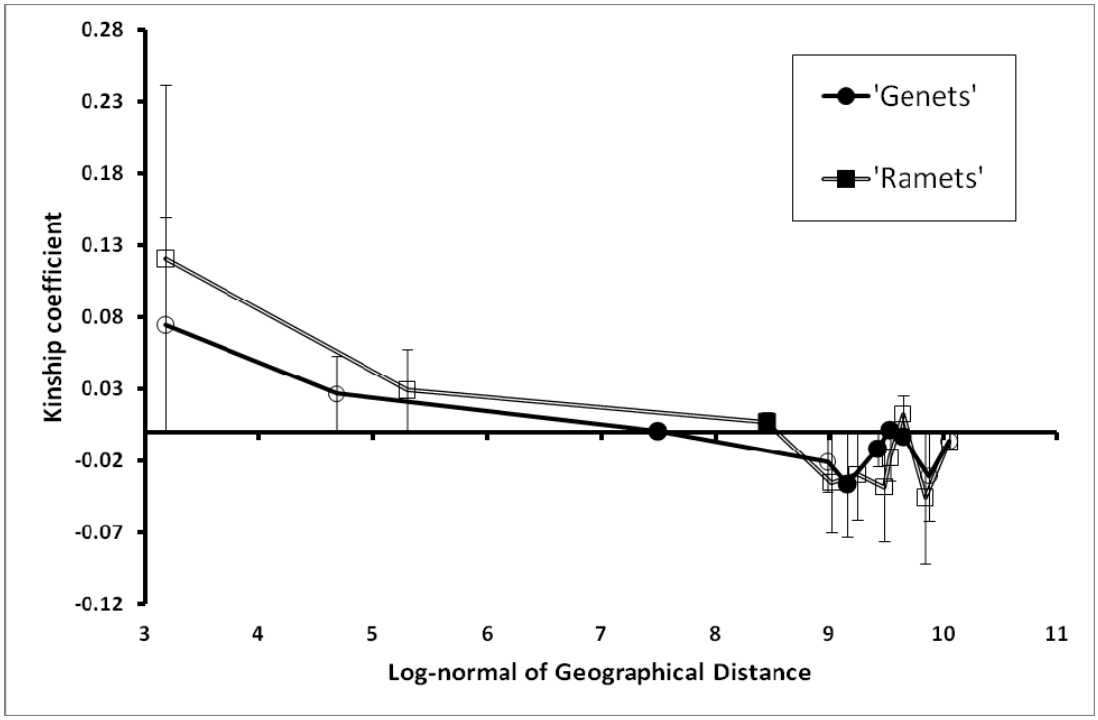

Figure 27 - Figure 6 Correlogram showing the spatial genetic structure at large scale of all the ramets and genets from Mayotte, with average Loiselle kinship coefficients over all loci (±SD) plotted as a function of the log-normal geographical distance. Significant kinship coefficients are indicated by empty symbols ($P < 0.05$).

3.3.5 Discussion

Evaluation of the genetic diversity plays a key role in determining population viability and the evolutionary potential of species (Endels et al. 2007; Frankel 1974; Allendorf et al. 2002; Hughes et al. 2008; Frankham 1995; Laikre et al. 2010; Aguilar et al. 2008). Several authors have emphasized the importance to consider the genetic factors in the investigations of extinction risk, or recovery plans for threatened taxa because in the long term, inbreeding and loss of genetic diversity may contribute to extinction in most wild populations (Frankham 2005; Bradshaw and Holzapfel 2001; Umina et al. 2005; Gienapp et al. 2008) and this is particularly true when it concerns naturally outbreeding species (Frankham 2005). The study based on 11 microsatellite loci gave us a comprehensive assessment of the genetic diversity of *V. humblotii* in Mayotte.

A mixed mating system in *V. humblotii*

The microsatellite markers revealed a relatively high average H_E of 0.331 over populations and all populations (but Sohoa) complied with Hardy Weinberg allelic frequency expectations (Table 2). The AMOVA revealed that the majority of the genetic diversity was found within populations rather than among populations (Table 3). All these results characterize species with an allogamous mating system (Fischer et al. 2000; Martin et al. 1997; Mekuria et al. 2002; Verma et al. 2007; Wang et al. 2005) in accordance with previous analyses of the reproductive system of *V. humblotii* which suggested a pollinator dependant system for reproduction (Gigant et al. in prep).

As previously demonstrated (Gigant et al. in prep), *V. humblotii* shows a mixed mating system (sexual and vegetative). The spatial organisation of clonality in the different populations confirmed a phalanx strategy in *V. humblotii* (Gigant et al. in prep). Clonality needs to be surveyed as it may have profound effects on the genetic diversity levels because it is expected to increase geitonogamous self-fertilization particularly in phalanx species (Vallejo-Marín et al. 2010; Fischer and Van Kleunen 2002). We found a high clonal genotypic diversity of *V. humblotii* at the scale of Mayotte (G/N = 0.73) despite an important maximal clonal patch size of 84 m (M'Tsamoudou) and a mean maximal patch

size of 19.3 m. *V. humblotii* clonality in Mayotte appears less important than in many other clonal plant species (for which mean genotypic diversity = 0.42 (Vallejo-Marín et al. 2010)). Clonal genotypic diversity for each population was not correlated with F_{IS} values (P = 0.99) and all populations (but Sohoa) were at HWE (Table 2). This shows more probably that clonality might not be sufficiently important to influence mating towards strong inbreeding following increased geitonogamy, which is limited also by scentless and rewardless flowers in accordance with the geitonogamy-avoidance hypothesis (Gigant et al. in prep; Dressler 1981; Jersakova and Johnson 2006; Jersáková et al. 2006; Johnson and Nilsson 1999; Johnson et al. 2004; Smithson and Gigord 2001). Inbreeding depression may also act against geitonogamous self-pollination success (Tremblay et al. 2005; Fischer and Van Kleunen 2002).

Allele losses as the main impact of fragmentation on genetic diversity in *V. humblotii*

Over the 136 'ramets' sampled across 9 remnant populations, 95 were 'genets' and each represented a unique multilocus genotype across Mayotte, witnessing the high level of diversity present. Microsatellite markers revealed relatively high levels of population variation with H_E ranging from 0.214 to 0.450 (Table 2). Even for very small remnant populations such as Chiconi, Choungui, Handrema, and Saziley (N ≤ 5 genets), for which we expected to find low levels of genetic variability due to strong genetic drift and inbreeding following founder effect (Shapcott 1995), results of gene diversity (H_E) and the FCA revealed that the small isolated populations were not genetically depauperate and exhibited a comparable genetic diversity than the larger ones.

Allelic richness was found to be correlated with the sample size of *V. humblotii* populations, while other parameters (H_O, F_{IS}) varied irrespective of the size of the populations. Regardless of the disturbances influencing the size of the populations, measures of allelic richness are considered more sensitive to population fragmentation than is gene diversity and are effective to detect loss of genetic variation in fragmented populations (Widmer and Lexer 2001) because elimination of rare alleles contributing little to heterozygosity (Cornuet and Luikart 1996). So the impact of

fragmentation identified through our descriptive parameters permitted to identify the loss of allelic richness as an impact of fragmentation following demographic bottleneck, but no loss of genetic diversity as measured by heterozygosity levels. Signs of recent genetic bottleneck can be witnessed by the occurrence of private alleles in most populations studied, with frequencies higher than 10 % (Table 2). A population in a mutation drift-equilibrium is expected to have a larger proportion of alleles with low frequencies. On the contrary, a population who has experienced a recent genetic bottleneck tends to lose rare alleles which consequently inflates the frequencies of the common alleles (Luikart et al. 1998). In eastern China, in the fragmented populations of *Berchemiella wilsonii* var. *pubipetiolata,* Kang (Kang et al. 2005) observed an absence of rare alleles associated to a reduction of allelic richness but without reduction of overall genetic heterozygosity which was interpreted as initial signs of deterioration of the population genetic dynamics.

Negative effects of fragmentation on genetic diversity are limited by clonal reproduction and reduced generations since fragmentation

Life history and ecological traits are important to define a plant susceptibility to genetic erosion in fragmented habitats (Aguilar et al. 2008). In our case, two main reasons can be proposed to explain the maintenance of genetic diversity in Mayotte populations: The clonal reproduction and the reduced generations since fragmentation.

Most *Vanilla* species, and *V. humblotii* in particular, are long-lived vines thanks to vegetative reproduction by natural stem cuttings. The vegetative reproduction may enable individuals to persist and maintains genetic diversity by reducing the probability of genet death (Cook 1983). Most fragmented forests retain genetic variation because trees in fragments are living relicts of predisturbance populations (Kramer et al. 2008). Similarly, vegetative reproduction provides the ability to counteract the extinction of small populations by preserving genetic variation and mitigating the effects of demographic stochasticity (Schaal and Leverich 1996). Aguilar et al. (2008) demonstrated that vines and epiphytes do not show significant negative effects of habitat

fragmentation. Wolf (2000) found in the self-incompatible orchid *Calystegia collina* showing a mixed mating system, no genetic impoverishment in small isolated population. On the opposite, the genetic variability of annual species can be quickly affected (Ouborg et al. 1999).

In clonal plants, genet immortality can potentially lead to unlimited persistence of established populations (de Witte et al. 2011; Thomas 2002), even if it is often difficult to estimate the exact age of clonal plants (de Witte and Stöcklin 2010). Klekowski Jr (1997) suggested that some genotypes might persist for hundreds or thousands of years vegetatively by reproducing indefinitely the genets. Recently, using molecular markers and modelling, Takahaschi et al. (2011) revealed that 10 000-year-old genets may be common in populations of saw palmetto (*Serenoa repens*) via vegetative sprouts. Genetic studies showed that cultivated vines of *Vanilla planifolia* have been propagated vegetatively by man from a single very old genet collected in Mexico in the early 1800s (Bory et al. 2008c; Lubinsky et al. 2008; de Witte and Stöcklin 2010). This shows that *Vanilla* has the capacity of sustaining 200 years old vegetative propagation without major signs of senescence.

The reduction in average heterozygosity per locus depends not only on the size of the population bottleneck, but also on the subsequent rate of population growth (Nei 1975). Therefore the allelic diversity seems to reflect more accurately the levels of genetic diversity within unstable population in the case of recent population reduction (Lowe et al. 2004; Lowe et al. 2005). Aguilar et al. (2008) showed that negative effects on plant genetic diversity are detected from 50-100 years of fragmented conditions. Given the 50-200 years old deforestation in Mayotte, this shows that the number of *V. humblotii* generations since fragmentation is probably not large enough to have generated important consequences on genetic diversity (Ehlers and Pedersen 2000), this being increased by low levels of sexual reproduction. We indeed found low natural fruit set (0.8 %) in Mayotte (Gigant et al. in prep) but in accordance with low values recorded for many leafy *Vanilla* species in America such as *V. planifolia* (about 1% fruit set) (Gigant et al. 2011a). Furthermore, low seedling recruitments were observed for this species (Schlüter 2002) and in *V. pompona*

(Householder et al. 2010). However the fruit set of *V. humblotii* is much lower than in the American leafy *V. chamissonis* (15%) (Macedo Reis 2000) and in Puerto Rico *Vanilla* leafless species: 14.5% (*V. dilloniana*) to 18.5% (*V. barbellata*) (reviewed in Gigant et al. (2011a)). Either 0.8 % fruit set is enough to generate high levels of genetic diversity, or this value represents the current fruit set whereas it used to be higher before fragmentation. In *Vanilla* as in most allogamous orchid species, fruit production is pollinator rather than resource – limited (Tremblay et al. 2005). High success rates of manual pollinations confirm this assertion for most orchids (Tremblay et al. 2005) as in *V. humblotii* (Gigant et al. in prep). Authors have demonstrated the indirect impact of fragmentation on plant diversity through the disruption of plant-pollinator interactions leading to increased pollen limitation (Aguilar et al. 2006; Andrieu et al. 2009). This could be the case for *V. humblotii* in Mayotte in those hardly fragmented populations, in which there might be in addition no suitable microsites for germination (no deep litter layer favouring seedlings) due to bad agricultural practices.

Therefore, the long-living character of *V. humblotii* individuals through vegetative propagation has allowed to retain the original levels of genetic diversity present before fragmentation. The limited generations elapsed in fragmentation conditions (increased by low fruit set) have further probably limited the negative effects on genetic diversity. All populations are indeed at HWE, showing no detectable homozygous excess (Table 2) which would be expected as a consequence of strong genetic drift or inbreeding following demographic bottleneck and population isolation. The only population with significant heterozygote deficiency is Sohoa (Table 2). We suggested that self-pollination through geitonogamy might be a plausible explanation for this homozygous excess (Gigant et al. in prep). The genetic consequences are however limited (F_{IS} = 0.086, Table 2) and concern only one marker (mVroCIR05) (Gigant et al. in prep). Other likely explanations could be local adaptation by selection or initial signs of genetic drift. The population is situated in one of the last extant primary mesophilous forest of low altitude from Mayotte (Pascal et al. 2001), where conditions are met to favour seedlings germination, biodiversity and particularly populations of

pollinators. It is probably the last large population of *V. humblotii* able to fully express its potential of vegetative and sexual reproduction. It might therefore be the one with the highest generation time since fragmentation as corroborated by private allele frequencies not exceeding 10% (Table 2). The ideal growing conditions may benefit to sexual reproduction but in return the genetic consequences of isolation of the populations, the limited gene flow with nearby populations (significant F_{ST} = 0.141 with the nearest population: Chiconi) will probably modify the genetic composition of the population towards a gradual locus fixation (by inbreeding or genetic drift), which might greatly reduce the ability of long-term survival of this population (Frankham 2005; Bradshaw and Holzapfel 2001; Umina et al. 2005; Gienapp et al. 2008).

Genetic structure and differentiation of the populations

A moderate mean differentiation across the nine populations from Mayotte was revealed *(*F_{ST} = 0.115), increasing for the 'ramets' dataset (F_{ST} = 0.162) as the presence of repeated genotypes in a population affects allelic frequencies and increases the derived F_{ST} values. Indeed all clones detected were specific to a population, in accordance with Ellstrand and Roose (1987) who already noticed the low probability that a clone can be repeated in different populations. The mean number of migrants *N*m (1.790) obtained from all pairwise comparisons is in accordance with the estimations realized for two American leafless *Vanilla* species *V. claviculata* (*N*m = 1.33) and *V. barbellata* (*N*m = 1.78) in Puerto Rico (Tremblay and Ackerman 2003). Our estimation obtained with the private allele method (*N*m = 0.575) is however lower. *N*m < 1 will favour genetic drift or local adaptation by selection, whereas *N*m > 2 prevents it (Tremblay & Ackerman 2003). As we will demonstrate, the intermediate gene flow revealed (0.575 ≤ *N*m ≤ 1.790) characterizes populations with an important variance in gene flow depending on the amount of the migration events varying in space and time (Tremblay and Ackerman 2003), resulting in a complex pattern of differentiation/connectivity for *V. humblotii* populations in Mayotte (Table 4). The genetic structuring proposed by the Geneland analysis revealed the presence of nine clusters highly concordant with the geographical localisation of the

individuals, although Structure revealed six clusters witnessing variable degrees of admixture for some populations. Mismatched results between the two methods are often reported (Braaker and Heckel 2009), but in our case, discrepancies concern evidence of admixtured populations or migrant individuals with a general pattern supported by the measures of gene flow (*N*m) and differentiation of the populations (F_{ST} values) if the cases of small populations (N ≤ 5) are treated cautiously given calculations based on allelic frequencies. We will discuss first the results obtained for geographically isolated island (Moya, M'Bouzi) or peninsula (Boungoudranavi) populations, and secondly those concerning Grande Terre populations.

Island populations: a combination of isolation by geographical barriers and colonisation events by LDD

Our F_{ST} analyses revealed that Moya on the island of Petite-Terre was significantly differentiated from all the other large populations of Mayotte (Table 4). Island populations are known to exhibit lower genetic diversity than mainland populations (Eldridge et al. 1999; Frankham 1997; Frankham 1998) due to founder events and reduced gene flow (Frankham 1997), as evidenced by the lowest genetic diversity observed in Moya (H_E = 0.214). A similar result was also revealed in leafless *V. barbellata* in Isla de Vieques island 20 km away from main Puerto Rico populations (Nielsen and Siegismund 1999). Interestingly, the Structure analysis clustered Handrema and Moya together (Figure 5), even when no prior population information was selected for the calculations (data not shown) which is in accordance with the non-significant F_{ST} value between the two populations (Table 4). This shows that *V. humblotii* from Petite Terre might have resulted from a recent colonization event from populations close to Handrema but the reverse way cannot be excluded.

The population of Boungoudranavi was significantly differentiated from all the large populations of Mayotte based on F_{ST} values (Table 4), which was supported by Geneland and Structure (Figure 5) results. Boungoudranavi is the southwestern-most population of Mayotte connected to Grande Terre by a fine strip of land of a few hundred meters. This part of Mayotte may be regarded as a peninsula

since it will become an independent island from Mayotte in the future. This geographical situation might be responsible for the observed genetic isolation of this population.

On the contrary, the island population M'Bouzi was not significantly differentiated from the other large populations of Mayotte (apart from Moya and Boungoudranavi, discussed above) (Table 4). Although the northern-most individuals of M'Bouzi constituted an independent cluster (Figures 4 and 5a), the two clustering analyses consistently revealed that several individuals shared coancestry with the geographical population of Saziley, 20 km farther south (Figures 4 and 5b). These individuals are probably migrants that have recently established by seeds from the population of Saziley (reverse migration route might also be possible). M'Bouzi is the only National Natural Reserve of Mayotte which is almost free of human disturbances since 1992. It is probable that the soil has begun to regenerate facilitating recruitment of new individuals.

Nothing is known about potential seed dispersers of *V. humblotii* but in America, seeds of the *Vanilla* with fragrant fruits are probably dispersed either by bats or bees (Gigant et al. 2011a). Three indigenous species of bats are present in Mayotte, *Chaerephon leucogaster* and *C. pusillus* and the large frugivorous *Pteropus seychellensis comorensis* well known to disperse seeds of native plants in western Indian Ocean islands (O'Brien 2011). Such LDD could be mediated by bats in Mayotte or by any animal capable of traveling such distances across sea.

Grande-Terre populations: a combination of IBD pattern below 10 km suggesting ancient forest connectivity, LDD events and isolation

The remaining Grande-Terre populations Handrema, M'Tsamoudou and Sohoa appeared as well defined independent clusters with Geneland, Structure (Figure 5) and F_{ST} data (Table 4). F_{ST} values were higher when considering the northernmost Handrema population (0.159-0.427) than the 3 southern populations from Grande Terre separated by less than 10 km (Choungui, M'Tsamoudou, Saziley,) (0.107 – 0.162) (Table 4). Interestingly, although M'Tsamoudou and Saziley appeared as different clusters, some individuals in these populations are admixtured with other populations

(Figure 5). Also, Choungui appeared as an admixtured population between Sohoa, Saziley and/or Chiconi, and Boungoudranavi (Figure 5) indicating genetic connectivity between these populations. Choungui population has potentially the best geographical position to undergo gene flow with southeastern (Saziley) and southwestern (Boungoudranavi) populations. This pattern is highly consistent with the IBD scenario revealed below 10km (Figure 6) which implies that neighbouring individuals and therefore neighbouring populations exchange more migrants than distant ones. This result extends our previous demonstration of IBD at fine scale in the Sohoa population (0-100m) (Gigant et al. in prep). The identification of a small carpenter bee *(Allodape obscuripennis*) as a potential pollinator was proposed to explain this IBD pattern at fine scale, with seed dispersal higher than pollen dispersal (Gigant et al. in prep). Spatial genetic structuring at shortest distances between individuals was also detected (<10 m) due to clonal patches (Gigant et al. in prep). The influence of clonal growth on spatial structuring can still be seen at large scale as it increases the IBD pattern in the first distance class intervals (Figure 6). The IBD pattern revealed for 1-10km is nevertheless surprising given the hostile agricultural landscape which should prevent animal-pollination between fragmented populations. More surprisingly, some distant populations were not differentiated by Structure, as for Chiconi and Saziley (Figure 5), and the Choungui population also showed clear admixture with distant Sohoa (Figure 5). Due to the long-lived clonal character of *V. humblotii*, these results might therefore, again, reflect an ancient situation (before fragmentation) when some close populations were still interconnected within large forest areas as the primary forest remnant patches were supposedly part of an extended forest system before deforestation (Guéneau 2006; Pascal et al. 2001)). On the other hand, seed dispersal could be implicated in gene flow between isolated populations as some seed mediated LDD events were indeed previously discussed for M'Bouzi / Saziley, or Moya / Handrema. In addition, localised geographical barriers in the hilly landscape of Grand-Terre might also be responsible for the lack of gene flow revealed between some neighbour populations such as Sohoa and Chiconi (Figure 5, Table 4).

Gene flow depends on the species but is probably variable both in space and time and may be unpredictable at large scale because it is greatly influenced not only by the reproduction mode but also by the life history of the populations and the surrounding landscape (Ellstrand 1992b, a; Nathan 2006; Nathan and Muller-Landau 2000; Nathan et al. 2008; Cain et al. 2000). This is particularly true at the scale of Mayotte, with a combination of a IBD pattern for distances lower than 10 km (with however possible localized geographical barriers) and LDD events (particularly for the colonization of Petite Terre and M'Bouzi from Grande Terre populations) giving a complex pattern of genetic connectivity / isolation in the remnant populations of *V. humblotii* in Mayotte. The long-lived character and overlapping generations in *V. humblotii* vines obviously mix ancient and recent gene flow scenarios. In order to better understand the life history of the populations of *V. humblotii* in Mayotte it will be relevant to complement our nuclear analysis by the addition of microsatellite or intergenic cytoplasmic markers. Their maternal inheritance and the low levels of recombination should permit to assess the evolutionary history of the populations over different spatial and temporal scales (Wang 2010; Braaker and Heckel 2009; Pease et al. 2009) with regards to the periods of geologic upheaval and the influence of other populations from the nearest islands of the archipelago.

3.3.6 Implications for the conservation of *V. humblotii* in Mayotte

The impact of fragmentation appeared genetically limited in *V. humblotii* populations from Mayotte given levels of genetic diversity and no evidence of strong inbreeding or genetic drift observed in these isolated populations despite very limited numbers of individuals. This indicates that the short term threat is probably anthropogenic due to habitat fragmentation and not genetic. Anthropogenic pressure is very important in Mayotte with a population multiplied by four in less than 30 years (Trouillard et al. 2009) accompanied by an increase of the infrastructure in lowlands, and the settlement of many slums in steep areas or along riverbeds (Audru et al. 2010). Bad agricultural

practices (burning) are witnessed in many areas where *V. humblotii* still occurs, with interspersed banana (*Musa spp)* or cassava (*Manihot utilissima*) cultivation. In just one year, we have indeed observed the almost complete disappearance of the individuals from Boungoudranavi at the lowest altitude as their support trees were burnt and replaced by banana crops (pers. obs.). Furthermore, the emergency is still present even in populations free of anthropogenic pressures with for instance infestations by introduced cochineal (*Conchaspis angraeci* Cockerell, 1893) of all the sampled individuals in M'Bouzi island (National Natural Reserve) in addition to predation of flower buds presumably attributed to introduced rats preying on soft succulent tissues of *V. humblotii*, in the absence of freshwater source on the island (pers. obs.).

V. humblotii may be considered a pioneer k-species according to which after recruitment of a few seedlings, the individuals would be able to colonize a site by vegetative reproduction. Indeed, *V. humblotii* is able to grow vegetatively on poor soil and in quite extreme temperatures even in degraded areas. Nevertheless long term viability relies on sexual reproduction which is essential to conserve an evolutionary potential to face environmental modifications (Ouborg et al. 2006). The long-term situation is therefore alarming because *V. humblotii* sexual reproduction appears very limited nowadays.

The best conservation strategy would be to preserve both large areas where sexual reproduction occurs more efficiently (probably the population of Sohoa), and as many small populations as possible. The important genetic diversity retained in the small populations indicates that conservation efforts should not ignore them because they should be considered as reservoirs of genetic variation, as private alleles were detected. Small populations (N < 5) could be preserved both *in situ* and in *ex situ* collections as they are the most directly threatened by anthropogenic pressure.

Following our sampling efforts, some individuals are already maintained *ex situ* in the BRC Vatel and *ex situ* preservation in Mayotte is scheduled for the individuals from the very small populations. *In situ* preservation of *V. humblotii* in M'Bouzi is urgently needed taking into account the introduced

rats preying the buds. It seems an emergency to eradicate them from the island to ensure natural sexual reproduction not only of *V. humblotii* but also many other species potentially threatened by their presence. Furthermore, the survival of the species in the island, given the high rate of cochineal infections, will probably require the introduction of genotypes from other genetically related and ecologically similar populations (Chiconi, M'Tsamoudou or Saziley could be options) to increase its evolutionary potential.

More broadly, forestry operations such as planting specific trees (*Acacia mangium*) and herbs (*Vetiver zizanoides*) are performed to restore soil properties (Izard et al. 1999) damaged by improper agricultural practices (strongly erosive) and to stop the formation of badlands in Mayotte (Audru et al. 2010). Interestingly, we have observed in the population of Sohoa many spontaneous individuals of *V. humblotii* growing on *A. mangium* where recently, there was likely at the same place only badlands. These are positive signs of the capacity of regeneration of the natural habitat where management measures of restoration are taken.

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CHAPITRE 4

CHAPITRE 4 : Etude de *Vanilla roscheri* en Afrique du Sud

4.1 Préambule

Le quatrième chapitre montre une étude de la reproduction et une évaluation des niveaux de diversité génétique de *Vanilla roscheri* en limite d'aire de distribution, sur les berges du Lac Sibaya, dans les forêts côtières du KwaZulu Natal (iSimangaliso Wetland Park, Afrique Du Sud), où il s'agit par ailleurs de la distribution la plus méridionale des orchidées vanilloïdes du continent Africain.

Sous la forme d'un article scientifique, les résultats d'une étude associant la mesure de la diversité génétique des populations et l'évaluation de la biologie de la reproduction sont présentés. L'objectif est de tester les conséquences de l'isolement des populations en distribution périphérique. Un total de 116 individus représentant quatre populations (distantes en moyenne de 2.7 km) de *V. roscheri* ont été recensées sur les berges du lac, e*t* sont évaluées à l'aide des méthodes de la biologie de la reproduction (évaluation des systèmes d'auto-incompatibilités à la reproduction et caractérisation de l'écologie de la pollinisation) et d'une étude génétique utilisant les marqueurs microsatellites développés.

Deux femelles abeilles allodapines (*Allodapula variegata*, et *Allodape rufogastra*) et une femelle abeille anthophorine (*Anthophorini* sp) ont été caractérisées comme étant les principaux pollinisateurs de *V. roscheri* à Sibaya, et sont probablement impliquées dans le taux de fructification exceptionnellement élevé (26.3%) pour une espèce non-spontanément autofertile du genre *Vanilla*. Malgré ce taux de fructification, étonnement, l'analyse génétique révèle une absence totale de diversité génétique associée à une homozygotie généralisée à l'ensemble des loci. Cette absence de diversité génétique reflète l'absence de flux de gène entre les populations de *V. roscheri* à Sibaya et les populations septentrionales, mais seul un goulot d'étranglement suivi d'une forte consanguinisation peut expliquer la perte totale de diversité génétique et l'homozygotie. Des mesures de conservation sont proposées pour protéger l'équilibre fragile des populations de Sibaya,

qui sont probablement très bien adaptées aux conditions écologiques de Sibaya, mais dont la base génétique du potentiel évolutif semble très limitée.

4.2 Article 4

Active sexual reproduction but no genetic diversity in the range-edge distribution of *Vanilla roscheri* Rchb. f. (Orchidaceae) in South Africa

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4.2.1 Abstract

Background In South-Africa, the wild leafless *Vanilla roscheri* (Orchidaceae) is distributed only on the banks of the Lake Sibaya in KwaZulu-Natal Coastal Forest in the iSimangaliso Wetland Park. Being also the southernmost *vanilloid* orchid in the Africa continent, the species is of high conservation priority which requires to gain basic knowledge on its reproductive biology and levels of genetic diversity.

Methods Mating systems were assessed by *in situ* breeding experiments and characterization of the species pollination ecology. Furthermore, the populations were exhaustively sampled and genotyped using 20 microsatellite markers.

Results Allogamous, the species depends on pollinators to achieve sexual reproduction. It is also autocompatible but spontaneous self-pollination is prevented by an efficient rostellum. The highest natural fruit-set reported for a non spontaneous self-pollinating *Vanilla* species (26.3%) was associated with numerous flower visitors including two female allodapine bees (*Allodapula variegata* and *Allodape rufogastra,* Xylocopinae) captured with pollinia stored on the hind legs, and one female anthophorine bee. We report also, interestingly, an unprecedented case of generalized homozygosity and absence of genetic diversity in Lake Sibaya populations.

Conclusions Given the high natural fruit-set of *V. roscheri* and the pollinator richness in Sibaya*,* the species should exhibit a high level of genetic diversity, but it was not the case. The complete homozygosity and lack of diversity is probably the consequence of the range-edge distribution of the species. Likely, the species has suffered from both strong bottleneck and inbreeding in the past. The implications in terms of conservation of the species in South-Africa are discussed.

Keywords: *Vanilla roscheri*, South-Africa, range-edge distribution, mating systems, genetic diversity, conservation.

4.2.2 Introduction

The genus *Vanilla* (Vanilloideae, Orchidaceae) comprises more than 100 species and shows a world-wide distribution throughout the tropics between the 27th north and south parallels. Twenty four species can be found in Africa and the South West Indian Ocean (SWIO) islands (Bory et al. 2008b; Portères 1954). In South Africa, there is remarkably only one Vanilloideae representative: the species *Vanilla roscheri* Rchb (Linder et al. 2005). This species is part of a peculiar group of 18 *Vanilla* species characterised by absent or bract-reduced leaves, forming the so-called Aphyllae section of the genus (Rolfe 1896; Portères 1954). This character has evolved as an adaptation to dry conditions at least three times in the genus history (Bouetard et al. 2010). *V. roscheri* is part of a monophyletic group of seven leafless species from the SWIO area that appeared 4.4 million years ago from African leafy ancestor species (Bouetard et al. 2010). This clade comprises *V. madagascariensis, V. decaryana, V. montagnacii* and *V. perrieri* endemic from Madagascar, *V. phalaenopsis* endemic from Seychelles, and *V. humblotii* from the Comoros archipelago (Portères 1954).

Although *V. roscheri* was classically described as present in Zanzibar, Pemba and the region of Daar-Es-Salam in Tanzania (Portères 1954), there has been other reports of this species in Kenya (La Croix and Cribb 1995), as well as further south in coastal Mozambique (Clarke 1995; La Croix and Cribb 1995; Krupko et al. 1954; Da Silva et al. 2004). *V. roscheri* was also recently described near Lake Sibaya in the iSimangaliso reserve in KwaZulu Natal (KZN) in South Africa (Ward 1984) where it was ranked second of 42 species in order of conservation importance after *Bonatea lamprophylla* in the rare, threatened and endemic orchid list of the reserve (Combrink and Kyle 2006). *V. roscheri* exemplifies the case of plant populations in dry tropical forest that are particularly threatened by habitat fragmentation, and are likely the most threatened primary forest (Hoekstra et al. 2005; Janzen 1988). Tropical dry thicket and dry forest in KwaZulu Natal are scarce because they represent the southernmost distribution of this vegetation distributed patchily on the littoral from Mozambique to South Africa, alternating between dune forests, tropical coastal forest and

grasslands. These vegetations belong to the Maputaland endemism center internationally recognized since it belongs to the Maputaland-Pondoland-Albany biodiversity hotspot which contains the iSimangaliso Wetland Park World Heritage Site, five RAMSAR sites and nine Important Bird Areas (Smith et al. 2008; Smith and Leader-Williams 2006).

As Sibaya represents the extreme southern range distribution of *V. roscheri* and also of the *Vanilla* genus in Africa and SWIO, it could represent an interesting case of adaptation to extreme ecological conditions (Stöcklin et al. 2009; Bliss 1962; Eckhart et al. 2011; Bridle and Vines 2007; Mägi et al. 2011; van Heerwaarden et al. 2009) and deserves special attention. *V. roscheri* was also designed 'flagship' and 'focal' orchid species in iSimangaliso (Combrink and Kyle 2006), witnessing its conservation importance and the lack of present information. We therefore assessed the distribution of the species around Lake Sibaya, surveyed its reproduction biology during two flowering seasons and investigated the levels of molecular genetic diversity using previously developed microsatellite markers (Bory et al. 2008a; Gigant et al. 2011b). These results will be essential to design appropriate conservation guidelines for this species which will help, more largely as a proxy 'focal' species, to highlight the issues and threats surrounding rare, threatened and/or endemic species in general (Combrink and Kyle 2006). This is also the first such study on *Vanilla roscheri* in South Africa, and is part of our current effort to study the evolution, diversity and life-history traits of leafless *Vanilla* species in the SWIO area (Gigant et al. 2011b; Gigant et al. in prep-a; Gigant et al. in prep-b).

4.2.3 Materials and Methods

Study site

The populations of *V. roscheri* sampled occur in KwaZulu-Natal Coastal Forest (Scott-Shaw and Escott 2011) on the banks of Lake Sibaya in KwaZulu-Natal, South Africa (Figure 1 and 2). The land adjacent to the lake where *V. roscheri* occurs is community owned while the lake, which is situated on the sea-ward margins of the Mozambique Plain, forms part of the iSimangaliso Wetland Park.

Figure 28 - Figure 1 African distribution of the leafy and leafless species of *Vanilla* genus (Krupko et al. 1954; Ward 1984 ; Beentje 1990; La Croix and Cribb 1995; Da Silva et al. 2004; Portères 1954b)**.** The study site of the leafless *V. roscheri* in South Africa is precised.

The Mozambique coastal plain is composed of recent and tertiary sands and Pliocene/Miocene beds overlaying Cretaceous mudstone. The soils are wind distributed grey sands. The mean annual precipitation is 1030mm with temperatures varying from 11.5 °C in July to 28.7 °C in January. *V. roscheri* occurs at four known sites, both within and on the margins of forest patches. These forest patches are characterized by tree species such as *Dialium schlechteri* (Zulu pod-berry), *Balanites maughamii* (torchwood), *Manilkara discolor* (forest milkberry), *Cleistanthus schlechteri* (bastard tamboti), *Craibia zimmermannii* (small craibia), *Croton* spp. and *Mimusops caffra* (red milkwood),

Hymenocardia ulmoised (red heartfruit) and *Aloe bainesii* (tree aloe) (Kyle and Ward 1995). There are also a number of other orchid species, both terrestrial and epiphytic, that occur in these forests.

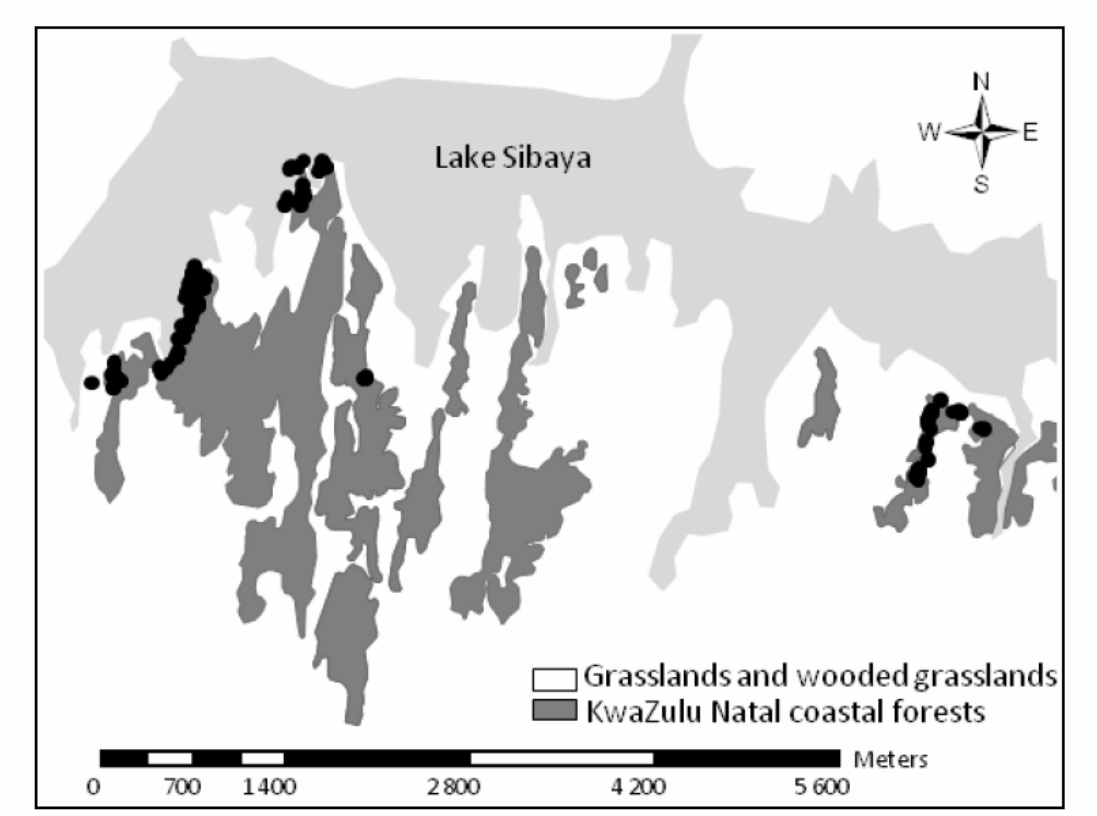

Figure 29 - Figure 2 Distribution of the four populations of *V. roscheri* (dark spots) on the border of Lake Sibaya in South Africa (Scott-Shaw and Escott, 2011).

Study species

V. roscheri is a reddish-brown leafless hemi-epiphytic vine 10 to 20 mm thick (Portères 1954). The species is described as mesoxerophytic by Portères (1954) and found between 0-100 m (La Croix and Cribb 1995). In north of South Africa, the flowering probably covers the rainfall summer season from November to March. The many-flowered racemes found on forest canopy are terminal or axillary. The large white 10 cm-long flowers are tainted with pink inside the labellum (Portères 1954; La Croix and Cribb 1995). The flowers are described as strongly and sweetly scented (La Croix and Cribb 1995). Two hairy rows up to 4 mm high, extend outward of the labellum and cover a narrow crest considerably shorter and invisible from outside of the flower, composed by two rows of digitated

lamellae (La Croix and Cribb 1995). The column measuring up to 2.5 cm long carries ventrally four yellow pollinia physically separated from the stigma by a rostellum. The straight capsules are 12-17.5 cm long (Portères 1954) and 7.5 mm wide (La Croix and Cribb 1995).

Reproduction biology

Floral measurements

In January 2010, 10 flowers from 10 inflorescences were randomly collected and stored in 70% ethanol prior to measurements. The floral characters were measured to the nearest 0.01 mm using a digital caliper (Figure 3). The mean length of an inflorescence (N = 48, 8 individuals), the mean number of flowers per inflorescence (N = 64, 11 individuals) and the number of inflorescences per individual (N = 11) were assessed.

Scent analysis

Floral volatiles were analysed using the solid phase micro extraction (SPME) technique (Zhang and Pawliszyn 1993) employing a gray StableFlex Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre, with a coating thickness of 50/30 μm from Supelco Co. (Bellefonte, PA, USA). The fibres were conditioned prior to use according to supplier's prescriptions (1 h at 270°C). Opened flowers were placed in a glass bell-jar (250 mL), closed by cotton at the large extremity, and by the SPME-fibre at the other end. The result reproducibility was ensured with four replicates. Fibres were exposed to the flower headspace for 2h30-5h (Table 1). Just after fibre exposition, GC/MS analyses were performed using a Hewlett Packard 5690N gas chromatograph, directly coupled with a Hewlett Packard 5973N mass spectrometer. Compounds were desorbed from the SPME-fibre in the GC injector (splitless injection mode) 10 min at 250°C. Volatiles were separated on a capillary SPB-5 non polar column (60 m x 32 mm; phase thickness 0.25 μm) with helium as carrier gas (0.7 mL/min). The GC oven was programmed to increase temperature from 60°C to 230°C at 4°C/min, followed by a stabilization at 230°C during 40 min. Mass spectra were produced with a current ionisation of 70eV, in a range of m/z 30-550 in the full-scan acquisition mode.

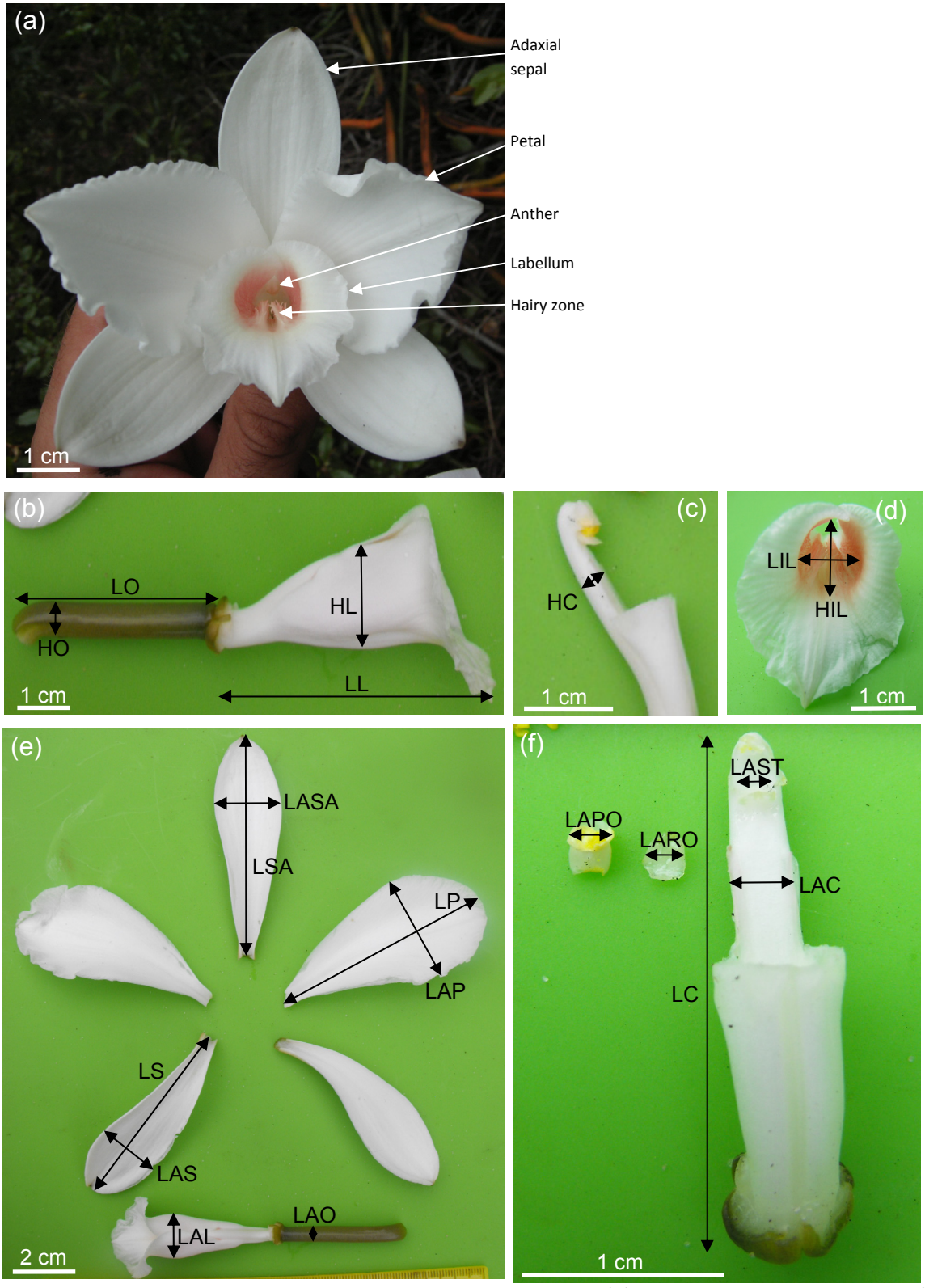

Figure 30 - Figure 3 Floral characters measured on *V. roscheri.* (a) Morphological description of a flower of *V. roscheri*; (b) Ovary length (LO) and height (HO); Labellum length (LL) and height (HL); (c) Column height (HC); (d) labellum internal width (LIL) and height (HIL); (e) Adaxial sepal width (LASA) and length (LSA), petal width (LAP) and length (LP), lateral sepal width (LAS) and length (LS), labellum width (LAL) and ovary width (LAO). (f) Width of the pollinia bag (LAPO), the rostellum (LARO), the stigma (LAST) and width (LAC) and length (LC) of the column.

Tableau 17 - Table 1 Floral volatile captures of *V. roscheri* in Sibaya. The presence of perfume was noted if it was detected before the introduction of the fiber.

Sample	Population	Fibre exposure time	Exposure date	Remarks
1	Population 1	09h00 - 11h30	27/01/2010	
2	Population 3	07h30 - 12h30	01/02/2011	
3	Population 1	08h45 - 12h45	18/01/2011	Light fragrance
4	Population 3	10h00 - 13h15	19/01/2011	Light fragrance

Pollinator observations

A hard-disk camcorder (Sony DCR-SR72E) mounted on a tripod with long-lasting batteries (NP-NH100 InfoLithium® and H series rechargeable batteries), and protected with a waterproof casing (Sony SPK-HCB marine sport pack) was used to observe the pollinators. Videotape sessions were conducted during the flowering season of January 2011. Each targeted flower was examined for pollen movement before and after the videotape sessions.

Insects visiting the flowers were collected and stored in 70° alcohol for further identifications. The pollen loads were collected from the silks and coloured using 1% fuschine and visualized using a Nikon Eclipse 50i microscope (Nikon, Melville, New York). The collected pollen was compared to the pollen of *Vanilla roscheri* flowers stored in 70°C alcohol.

Natural fruit-set

The mean natural fruit set was estimated by averaging the total number of fruits per the total number of flowers produced (estimated by the floral scars) in two flowering seasons in January 2010 and in January 2011 (64 inflorescences, 24 individuals).

Breeding systems and auto-incompatibility tests of *V. roscheri*

Compatibility systems in *V. roscheri* were evaluated by pollination experiments over two flowering seasons. Except for the self-fertility tests, no more than 1-3 breeding tests per inflorescence were realized to avoid limitation of fructification due to resource allocation. Self-fertility was evaluated by the exclusion of insect visits using insect-proof bags on 16 virgin flowers from 8 individuals. Auto-compatibility was tested by hand self-pollination using 25 flowers from 13 individuals. The cross-

pollination treatments were performed on 31 flowers from 13 individuals, by collecting the pollinia with a toothpick from an open unpollinated flower and placing it on the stigma of an unpollinated flower of another individual (Nielsen 2000). After treatment, the inflorescences were protected by an insect-proof bag. Abortion/success was recorded 6-15 days after pollination experiments. The fall of the faded flower characterized the unsuccessful pollinations and the swelling of the ovary successful ones (Lecomte 1901; Shadakshari et al. 2003). Comparisons between self and cross-pollination treatments were performed using Fischer exact test available in the software R 2.12.2 (R Development Core Team 2010).

Genetic analyses

Sampling of *V. roscheri* in the Sibaya population

In order to extract the maximum genetic diversity, an exhaustive sampling of the individuals found on different supports were employed, but given the difficulty to distinguish between stems which have arisen vegetatively from those derived from seedlings, an inter-sampling of 5 m was defined for individuals in continuity on different supports. Twenty centimetres of stem or young vegetative buds were sampled and stored in silica gel for DNA analyses. Following this strategy, 116 samples were collected and geolocalized in four populations, characterized as small and geographically isolated assemblages of plants (Figure 2).

DNA isolation and genotyping

DNA extractions were made using the CTAB protocol (Risterucci et al. 2000) adapted for 1g of lyophilized plant material. Nineteen markers developed from leafless *Vanilla* species (Gigant et al. 2011b) and one from *V. planifolia* (Bory et al. 2008a) were used to detect genetic polymorphism in *V. roscheri*. In addition to Sibaya individuals, *V. roscheri* accession CR0810 collected in Zanzibar and conserved in the BRC Vatel (Roux-Cuvelier and Grisoni 2010) was also genotyped. The PCR conditions described in Gigant et al. (2011b) were the same for all the markers facilitating the multiplexing.

4.2.4 Results

Floral characteristics of *V. roscheri*

In mean, an individual of *V. roscheri* carried 6.9 (± 5.7) inflorescences and the inflorescences measured 32.3 (± 7.2) cm and carried 13.9 (± 8.3) flowers.

Tableau 18 - Table 2 Mean (± SE) of the floral characters measured on *V. roscheri* in millimetres (N = 10).

Floral segments	Length	Width	Height
Ovary	40.5 ± 4.7	5.2 ± 0.3	4.6 ± 0.2
Adaxial sepal	66.1 ± 4.1	20.8 ± 1.2	NA
Lateral sepal	64.4 ± 5.6	20.9 ± 1.6	NA
Lateral petal	67.4 ± 4.0	32.2 ± 1.6	NA
Labellum	53.1 ± 3.4	12.4 ± 1.3	13.0 ± 1.2
Column	25.3 ± 1.2	2.8 ± 0.2	2.3 ± 0.1
Stigma	NA	1.6 ± 0.3	NA
Rostellum	NA	1.9 ± 0.1	NA
Pollinia bag	NA	2.5 ± 0.2	NA

Reported in Table 2, the quantitative characterization of the floral features of *V. roscheri* were in accordance with Portères' description of *V. roscheri* (1954), with petals similar in length to sepals but 61% wider. Our results revealed that the rostellum covers the entire stigmatic surface. Although a floral scent was detected in the field in two of the four flowers tested (Table 1), the SPME analyses failed to detect any volatile compounds.

Pollination ecology

The breeding system experiments suggest that *V. roscheri* is allogamous but self-compatible, since the fruit sets were as high for the cross pollinations as for the self-compatibility tests (hand self-pollinations) and not significantly different ($P > 0.5$). None of the autonomous self-pollinations excluding pollinators set fruits (Table 3) because of a large rostellum covering the entire stigmatic surface and preventing autonomous self-pollination. This indicates a pollinator-dependant system for reproduction because.

Tableau 19 - Table 3 Fruit set in percentage obtained from breeding experimentations with the number of flowers used each year.

	2010	2011	Fruit set
Pollinator excluded	6	10	0.0 %
Self-pollination	6	19	64.0 %
Cross-pollination	7	24	71.0 %

The mean natural fruit set averaged over two flowering seasons was estimated at 26.3% (1660 flowers). The lifespan of *V. roscheri* flowers was one day with flowers open from sunrise up to sunset. Night pollination hypotheses were therefore excluded from our investigations and the recording efforts were concentrated on day pollination observations.

Tableau 20 - Table 4 Pollinator spectrum of *V. roscheri* in Sibaya reported by 32 hours of videotape sessions in January 2011. The contact visits were counted when the visitor landed on the flower. The visit rates and mean duration of each contact visit are indicated.

Visitors	Contact visits	Visit rate (visits / h)	Mean duration (s)
Hymenoptera [a]			
Apidae			
Apinae Anthophorini			
Anthophora Friese sp. */ Amegilla* Latreille sp. (♀) [b]	2	0.06	17 ± 7
Xylocopinae Allodapini			
Allodapula variegata Smith (♀)	1	0.03	339
Allodape rufogastra Lepeletier and Serville (♀)	3	0.09	36 ± 16
Lepidoptera [c]			
Macrolepidoptera			
Papilionoidea			
Papilionidae sp. 1	1	0.03	3
Papilionidae sp. 2	1	0.03	2
Pieridae sp. 1	3	0.09	3 ± 1
Pieridae sp. 2	2	0.06	5 ± 3
Lycaenidae sp. 1	1	0.03	3
Diptera			
sp. 1	3	0.09	717 ± 936
sp. 2	3 [d]	0.09	104 ± 75
Colleoptera [e]			
Curculionidae sp.	1	0.03	201

[a] Identifications by Pr. Connal Eardley, Plant Protection Research Institute (PPRI), Pretoria, South Africa.
[b] Identification uncertain (insects not captured).
[c] Identifications by Antoine Franck (Cirad), Réunion, France.
[d] One visit comprised simultaneously two individuals on the same flower.
[e] Identification by Didier Pastou (FDGEDON), Réunion, France.

Recordings (32h) spanned over 8 days of observation between 7-8 am and 13-14 pm. Several insect visitors were observed during the recordings for which all the contact visits are reported (Table 4), but only three were associated with systematic pollen movements at the end of the recording session: *Allodapula variegata, Allodape rufogastra* and the Anthophorini.

One female *Allodape rufogastra* and two females *Allodapula variegata* were captured visiting the flowers of *V. roscheri* with pollinia stored on the tibia of the hind legs (Figure 4A and 4B, respectively) and the specimens were deposited (PPRI, South Africa). The microscopic observations revealed a single type of pollen, with double-walled, neither pore nor ornamentation, similar to the pollen of *V. roscheri* (data not shown). Screen captures of the anthophorine bee video showed two oblong pollinia of heterogeneous form on the mesonotum (Figure 4C) which may correspond with *V. roscheri* pollinia. Among the other contact visits, only one Lepidoptera (Pieridae sp. 2, 2 visits) and Diptera sp. 1 (3 visits) could not be excluded absolutely as potential pollinators, since their visits were within the same day as the allodapine or the anthophorine visits and a pollen movement was verified at the end of the video session. We noticed the almost omnipresence of ants, midges and also crab spiders (Tomisidae).

The allodapine visit duration varied greatly depending on the species (Table 4). Although shorter duration visits of the anthophorine bee, the bees landing on the labellum penetrated immediately inside the lip of *V. roscheri* flower. A bustle movement of the bee abdomen from left to right shaking the flowers, indicated that the anthophorine bee could be highly efficient to involve a pollen movement. On average (± SE) the visit rate was relatively low with 0.08 ± 0.08 visit per hour. Most of the visits were provided by Lepidoptera sp., but they remained the shortest time in contact with the flowers (Table 4), perhaps just enough time to taste the inside of the lip and realize the lack of nectar. Diptera sp. could remain much longer on the flowers (Table 4) but the duration was highly variable (SE = 936 sec) and most of the contact visits were not necessarily associated with a penetration in the labellum.

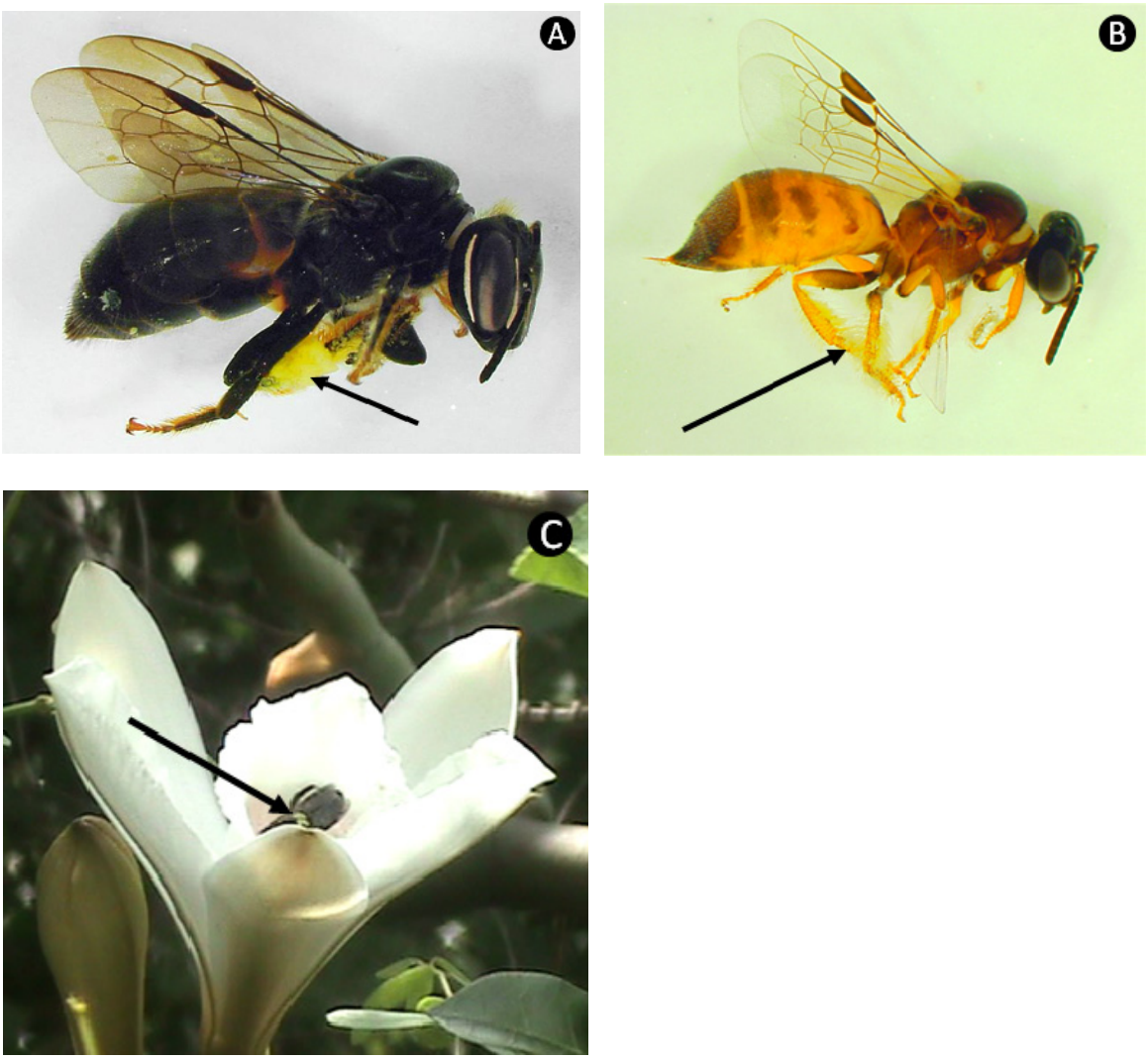

Figure 31 - Figure 4 Potential pollinators of *V. roscheri.* (A) *Allodape rufogastra* and (B) *Allodapula variegata* captured visiting a flower of *V. roscheri*; (C) Screenshot of an Anthophorini large bee visiting *V. roscheri* and carrying pollinia on the mesotonum. The positions of pollen loads (A and B) and pollinia (C) are precised by a black arrow on the picture (Credits photography A and B: Antoine Franck).

Population sampling and genetic diversity of *V. roscheri*

Most frequently, *V. roscheri* individuals were in large patches in continuity in the same population, in accordance with the predominant vegetative propagation characterized in many *Vanilla* species (as reviewed in (Bory et al. 2010; Gigant et al. 2011a)). Nevertheless, in underwood many individuals also appeared completely isolated from the large patches. Young seedlings were also found (data not

shown). The nearest populations were a distance of 700 m apart and the mean distance between each population was 2.7 km (Figure 2).

Among the 16 microsatellite loci showing repeatable amplifications, 69% (11) were polymorphic in *V. roscheri* (including CR0810) but remarkably, all the 116 individuals from the four Sibaya populations were monomorphic and homozygous for the 16 loci (Table 5). Comparatively, based on 11 polymorphic loci, seven loci were heterozygous in CR0810. A total of 29 alleles were revealed of which seven were private alleles to the Sibaya population of *V. roscheri* (Table 5).

Tableau 21 - Table 5 Genotypes of the 116 individuals of *V. roscheri* populations of Sibaya (South Africa) and CR0810 collected in Zanzibar Island given in nucleotides for 20 microsatellite loci. The non repeatable genotypes are precised (-).

Locus	Sibaya	CR0810
mVroCIR01 *JN222572*	140	132
mVroCIR03 *JN222573*	350	344 350
mVroCIR04 *JN222574*	204	206
mVroCIR05 *JN222575*	205	203 205
mVroCIR06 *JN222576*	196	192 198
mVroCIR08 *JN222577*	198	198 200
mVroCIR09 *JN222578*	239	227 239
mVroCIR10 *JN222579*	210	210
mVroCIR11 *JN222580*	180	172
mVhuCIR03 *JN222562*	128	128
mVhuCIR04 *JN222563*	150	150 174
mVhuCIR06 *JN222564*	-	-
mVhuCIR07 *JN222565*	-	-
mVhuCIR08 *JN222566*	220	220
mVhuCIR09 *JN222567*	206	198 202
mVhuCIR10 *JN222568*	216	216
mVhuCIR11 *JN222569*	270	275
mVhuCIR12 *JN222570*	-	-
mVhuCIR14 *JN222571*	-	-
mVplCIR031 *EF486655*	398	398

4.2.5 Discussion

An efficient pollinator-dependent sexual reproduction in Sibaya populations of *V. roscheri*

Hand-crossing revealed that the species is autocompatible (Table 3), but spontaneous self pollination is prevented by the presence of the rostellum covering the entire stigmatic surface (Tables 2 and 3). Therefore, the sexual reproduction of the species is pollinator-dependent as in most *Vanilla* species (Gigant et al. 2011a).

The entomofauna visiting the flowers of *V. roscheri* is species-rich with 11 visitors in four insect orders detected in Sibaya (Table 4). Among the visitors of *V. roscheri*, three bees can be considered as potential pollinators since they were always associated with pollen movements.

The bustling behaviour of the anthophorine bee, its size and the observation of pollinia on its mesonotum, make it a good candidate as *V. roscheri* pollinator. Anthophorine bees are robust, fast-flying, long-tongued bees nesting in the soil (Corlett 2004), known as nectar-seeking bees, they visit a large variety of both reward and rewardless flowers worldwide in several plant families. The paleotropical genus *Amegilla* pollinates various flowers but its role as pollinator is most important in the forest understorey in the Oriental Region, (Momose et al. 1998; Kato et al. 1991; Kato 1996; Kato et al. 1993; Sakai et al. 1999), with pollination reports on Zingiberaceae, Costaceae (Kato et al. 1993; Sakai et al. 1999) and other taxa of odorless flowers with nectar (Momose et al. (1998) and see the review of Corlett (2004)). Several reports imply that anthophorine bees pollinate orchids but few of them characterize these bees as main and effective pollinator. In South Africa, anthophorine bees were recorded as effective pollinators in two nectar producing orchids (*Disa versicolor, Satyrium erectum* (Van der Cingel 2001)) and in the nectarless *Disa racemosa* (Johnson 1994)). Nevertheless in most of reports, *Xylocopa* species, Bumblebees and *Trigona* bees were the main pollinators of flowers also visited by the anthophorine in Africa (*Eulophia cristata* (Van der Cingel 2001)), America (*Spiranthes diluvialis* (Van der Cingel 2001)), Asia (*Cymbidium insigne* (Van der Cingel 2001)) and Australia (*Dendrobium monophyllum* (Bartarau (1993) as cited in Van der Cingel (2001)). Given the

large pollination spectrum involving anthophorine bees, their role in the pollination in the nectarless food-deceptive flowers of *V. roscheri* is quite likely.

The capture of female allodapine bees with loads of *V. roscheri* like-looking pollen on the tibia of the hind legs suggests that the bee collect the pollen for brood provisioning. In female allodapine bees, the collected-pollen is the substrate for oviposition which then supplies their eggs and larval instars (Tierney et al. 2008; Wilson 1971). Since a part of the collected pollen is likely not available for pollination, allodapine bees are considered as pollen-thieves and they have been described as responsible for a reduction of the reproductive success of *Aloe maculata* (Aloeaceae) (Hargreaves et al. 2010). Nevertheless in *Bergeranthus multiceps* (Aizoaceae), where they have been observed as having as well their bodies covered with pollen by the act of collecting, female *A. variegata* was identified as the main pollinator of the plant (Peter et al. 2004). The only available reports on the pollination of orchids by pollen-collecting insects concerns the primitive genera (Freudenstein et al. 2004; Cameron et al. 1999) *Apostasia* (Apostasioideae) (Dressler 1986) and *Cleistes* (Vanilloideae) (Gregg 1991) with powdery pollen or loose pollen masses . These pollen structures may facilitate collecting behavior by insects contrary to solid-type pollinia of most higher orchids (Johnson and Edwards 2000). In *V. roscheri* the soft and loose character of the pollen masses and its belonging to an old lineage of orchids is compatible with the observed pollen collecting behavior by allodapine bees. Similarly, in Mayotte (Comoros Archipelago) an allodapine bee (*Allodape obscuripennis*) was also identified as the most frequent visitor of *V. humblotii* flowers, but no evidence of pollen collection or movement was detected there (Gigant et al. in prep-a). Remarkably in South Africa, the two types of pollinators suspected to pollinate *V. roscheri* were identified as pollinators of *B. multiceps*, with *A. variegata*, carrying exclusively *B. multiceps* pollen, identified as the primary pollinator, and among the several insect visitors, two anthophorines *Amegilla punctifrons* (Walker) and *Amegilla spilostoma* (Cameron) were also identified (Peter et al. 2004).

The natural fruit set of *V. roscheri* (26.3%) detected in South Africa, was the highest reported for a non spontaneous self-pollinating *Vanilla* species. Comparatively, the range of fruit set for such American species varied from 0.001% in *V. planifolia* to 18.2% in *V. barbellata* (as reviewed in Gigant et al. (2011a)). The only available data for African *Vanilla* species are well lower : 0% in *V. crenulata* (Johansson 1974, as cited in Soto Arenas and Cameron (2003)); 0.8% in *V. humblotii* (Gigant et al. in prep-a) and 1% in *V. madagascariensis* (Perrier de la Bâthie 1934). The high fruit set recorded for *V. roscheri* witnesses the effectiveness and abundance of visitors. Either the allodapine collecting behaviour allows much more pollination events than it could be expected for a pollen thief or the anthophorine bees are particularly effective to achieve the sexual reproduction. Nevertheless, the combined contribution of the two visitors may also be responsible for the high fruit set reported.

The flowers of *V. roscheri* in the field were heterogeneous in terms of fragrance. Such pattern has already been described for *V. pompona* in Peru (Householder et al 2010). Intraspecific variation in production and composition of floral fragrance was already reported by Tremblay et al. (2005) and related to food deceptive orchids (Salzmann et al. 2007). SPME analysis unfortunately failed to detect any volatile compounds maybe because these were at very low concentrations and perhaps given the time elapsed between field scent collection and laboratory SPME analyses. Given the large visitor spectrum (Table 4) of *V. roscheri* flowers, their fragrance may also be invoked as responsible for the high fruit set recorded in Sibaya (Majetic et al. 2009; Schiestl et al. 2010). In *Vanilla* species, the American species *V. chamissonis* is characterized by strongly fragrant flowers and a high fruit set (15%) (Macedo Reis 2000; Gigant et al. 2011a) whereas the non fragrant flowers of *V. humblotii* were associated with a low fruit set (0.8%) (Gigant et al. in prep-a). Even though not well documented, the labile character of floral signals of deceptive orchids is probably related to frequency-dependent selection due to the learning of pollinators that could avoid deceptive morphs (Schiestl 2005).

An absence of genetic diversity in *V. roscheri* populations in Sibaya

The genetic analyses of the 116 samples of *V. roscheri* in Sibaya highlighted a total absence of genetic diversity and an unprecedented case of complete homozygosity for the 16 amplified microsatellite markers (Table 5).

The informativeness of the markers could be questioned given the absence of genetic diversity in Sibaya but a single individual of *V. roscheri* from Zanzibar was enough to increase to 69% the number of polymorphic markers (Table 5). Microsatellite loci are well known to be the best suited markers for population genetic analyses given their hyper-polymorphism due to high slippage mutation rates (Halkett et al. 2005; Gitzendanner et al.). In *V. humblotii* populations from Mayotte, 11 markers among the 16 used in *V. roscheri* were polymorphic and showed neither linkage disequilibrium nor null alleles, even at small population scale witnessing their strong informativeness (Gigant et al. in prep-a; Gigant et al. 2011b). Furthermore, these markers were transferable and polymorphic in other *Vanilla* species, American and African leafy and African leafless species, notably *V. madagascariensis* for which, except the locus mVhuCIR03, the loci tested here were all polymorphic (Gigant et al. in prep-a). Therefore, all the four discrete populations of *V. roscheri* in Sibaya can be considered as sharing an identical multilocus genotype.

The results in Sibaya populations of *V. roscheri* are in accordance with a reduction of the genetic diversity given the range-edge distribution of the species. In South Africa, *V. roscheri* is found in KwaZulu-Natal Coastal Forest biome, which occurs in small patches on the landward side of the dune cordon along the KwaZulu-Natal coast, and extends from Southern KwaZulu-Natal to beyond the Mozambique border towards Tanzania where it shows a stronger tropical nature. The whole distribution of *V. roscheri* follows that of KwaZulu-Natal coastal forests and its reaches its southernmost distribution in Sibaya, but there are huge gaps in *V. roscheri* distribution and it is not found elsewhere in KwaZulu Natal where this vegetation type occurs (B. Church, pers. com.). The moist conditions of lake Sibaya might have favoured the establishment and growth of *V. roscheri*. The

nearest population of *V. roscheri* was described northernmost at 140 km in Inhaca Island (Mozambique) (Ward 1984; Krupko et al. 1954). In the literature, several authors report the loss of genetic diversity in marginal populations ((van der Merwe et al. 2010; Beatty et al. 2008; Silvertown 2008) and see the review of Johannesson and André (2006)). Extreme cases of total absence of genetic diversity in isolated populations can even be observed with a single genotype present as shown for *Eucalyptus globules* in North of Australia (Vaillancourt et al. 2001), or *Orthilia secunda* in Ireland (Beatty et al. 2008). These instances witnessed a dramatic loss of genetic diversity at the edge of species range, but the single genotypes revealed were heterozygous and clonality was retained as the sole mode of reproduction in these isolated populations. Here, we report an unprecedented case of total absence of genetic diversity but complete homozygosity for all microsatellite markers in *V. roscheri* from Sibaya.

One genotype entirely homozygous in Sibaya

The finding of a homozygous genotype is contradictory to several previous genetic analyses realized in closely related allogamous species of the *Vanilla* genus, given the floral features (rostellum) and the preferential outcrossing behaviour revealed. Expected heterozygosity (H_E) revealed by 11 microsatellite markers across *V. humblotii* (95 individuals) from Mayotte, varied from 0.08 to 0.58 (Gigant et al. in prep-b). Most populations complied with Hardy Weinberg frequencies expectations, as in Puerto Rico where 5 and 6 isozyme loci (over 7) were heterozygous in the leafless American *V. barbellata* (28 individuals) and *V. claviculata* (21 individuals) respectively (Nielsen 2000). Even in cultivated *V. planifolia*, for which most of the available material is derived from cuttings of a single clone (Duval et al. 2006; Minoo et al. 2007; Bory et al. 2008c), two microsatellites over 13 (comprising mVplCIR31 used here) were heterozygous (Bory et al. 2008a), although this species was described as suffering from strong inbreeding in natural conditions (F_{IS} values close to 1) (Soto Arenas 1999). Finally, it is noteworthy that the *V. roscheri* specimen from Zanzibar studied displayed 7 heterozygous microsatellite loci of the 16 studied (Table 5).

Strong bottleneck and inbreeding

Only inbreeding can therefore explain the occurrence of a homozygous genotype in Sibaya. Strong inbreeding is expected particularly if a population has experienced a major demographic bottleneck and reduced gene flow and two scenarios might be suggested: (i) an ancient extended population connecting Sibaya and northernmost populations of *V. roscheri* has undergone an important fragmentation isolating Sibaya population, or (ii) Sibaya populations derived from the establishment of few or one propagule by a long distance dispersal event from a northern population.

Given the absence of genetic diversity in Sibaya, the contributions of sexual and asexual reproductions are undistinguishable. The species can easily reproduce vegetatively (pers. obs.) but the high fruit set, the discrete distribution of the four Sibaya populations together with the discovery of seedlings, show that sexual reproduction is active in Sibaya. Like many *Vanilla* species*, V. roscheri* should reproduce both sexually and asexually, with clones probably aggregated in a phalanx distribution as it was demonstrated in *V. humblotii* in Mayotte (Lovett-Doust 1981; Gigant et al. in prep-a; Gigant et al. in prep-b). This clonal architecture enhances the opportunity of mating between related genotypes (Charpentier 2002; Reusch 2001) and was probably implicated in the inbreeding. As *V. roscheri* is autocompatible, the geitonogamous matings are allowed. Signs of inbreeding were indeed detected in the Sohoa population of the autocompatible *V. humblotii* (Lovett-Doust 1981; Gigant et al. in prep-a; Gigant et al. in prep-b). Strong inbreeding was also detected in *Vanilla planifolia* in Mexican forests due to a strong clonal patchy distribution (Soto Arenas 1999).

Whether strong inbreeding occurred in a northern population or directly in Sibaya cannot be ruled out. If in Sibaya, it must have been followed by inbreeding depression and purging of the deleterious genetic load to reduce the population to a single adapted genotype. Inbreeding depression ie the reduced fitness of inbreeds offspring, is due to the expression of the recessive deleterious alleles at the homozygous states (Charlesworth and Willis 2009; Charlesworth and Charlesworth 1987; Husband and Schemske 1997). Lower fitness due to inbreeding is expressed through a number of

plant traits such as in germination (Sheridan and Karowe 2000), biomass (Vange 2002), survival (Charlesworth and Charlesworth 1987), and reproduction (Husband and Schemske 1997), but the purging of inbreeding depression through the elimination of deleterious mutations by natural selection, i.e the benefit of loss of the genetic load (Reed et al. 2003) has probably resulted in the emergence of a well adapted genotype in Sibaya. Many examples of plants showing specific adaptation to extreme range habitats are available (Stöcklin et al. 2009; Eckhart et al. 2011; Larcher et al. 2010) and this could be the case for *V. roscheri* in Sibaya. On the other hand, it could simply be a population established from a single homozygous migrant from an inbred northernmost population.

A counterintuitive situation in Sibaya

Given the rich entofauna visiting flowers and collecting pollen, and the highest value of fruit set reported for a non spontaneous self-pollinating *Vanilla* species, high levels of genetic diversity were expected in Sibaya populations of *V. roscheri*. On the contrary, the genetic analyses revealed a total absence of variability even going so far to a complete homozygosity. To our knowledge and whatever the marker employed, it is the first report of a complete loss of genetic diversity and homozygosity in a wild plant population, particularly in an allogamous species with such a high fruit set. Interestingly, the reverse situation was observed previously in *V. humblotii* populations from Mayotte for which high levels of genetic diversity were observed even in very small populations coupled with low value of fruit set (0.8%), no pollen movements and a low diverse entomofauna of visitor (Gigant et al. in prep-b; Gigant et al. in prep-a). This paradoxical situation in Mayotte was attributed to the coercive action of the maintenance of the genetic diversity by vegetative reproduction and low pollinator/visitor populations influenced by a highly degraded surrounding landscape. The results reveal the interest of the complementary approach associating reproductive biology and genetic analyses. Indeed, without genetic analyses, we would not have been able to detect the threats on *V. roscheri* in South Africa.

4.2.6 Implications for the conservation

The integrated approach of molecular and ecological data has provided a great insight into the situation of the Sibaya populations of *V. roscheri* (Gaston 2009; Geber 2008). Despite the absence of genetic diversity, the species is probably as viable as large populations which could result from local adaptations and emphasizing its potential value for conservation (Lammi et al. 1999). Since long-term conservation of species depends upon the conservation of peripheral populations (Lesica and Allendorf 1995), *V. roscheri* Sibaya populations is a top priority for conservation of the species.

The next step of the investigations will necessitate the identification and study of northernmost *V. roscheri* populations, if they are still present, to test our hypotheses on the origins of the population in Sibaya. In Zanzibar, the primary natural forest has almost completely disappeared therefore it seems unlikely to find new *V. roscheri* populations there. The most recent reports on the presence of *V. roscheri* were in 1990 in N'Gezi forest on Pemba island (Tanzania) where several populations are described (Beentje 1990). In Mozambique, the large *V. roscheri* population described in 1954 in Inhaca island (Maputo) might not still be present (Krupko et al. 1954), but more recently, *V. roscheri* was reported to occur in Inhambane (Da Silva et al. (2004) and from a collection in 1980 cited by La Croix and Cribb (1995)).

These populations could represent opportunities for increasing the genetic diversity in Sibaya, since given its current genetic state, the evolutionary potential of *V. roscheri* to face environmental change seems dangerously limited. On the other hand, if the situation observed is the result of a long term evolutionary process which has favored the emergence of one genotype particularly well adapted to the local conditions of Sibaya, importing foreign variability could potentially affect the fragile balance of Sibaya populations by introducing deleterious alleles. Nevertheless, urgent, simple and efficient *ex situ* conservation strategies can already be set up to protect the single homozygous genotype from Sibaya from any drastic disappearance which may be caused by a low ability to face environmental

modifications, since, even in the apparently preserved surrounding landscape of Sibaya, several anthropogenic pressures are important threats for the natural habitat (Von Maltitz et al. 2003; Smith et al. 2008; Smith and Leader-Williams 2006).

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CHAPITRE 5

CHAPITRE 5 : Discussion générale et conclusions

Les objectifs principaux de ce travail de thèse consistaient à identifier les populations naturelles de deux espèces de vanilliers menacées, *V. humblotii* à Mayotte et *V. roscheri* en Afrique Du Sud, et de rendre compte de leur diversité génétique et de leur biologie de la reproduction afin de suggérer des mesures de conservation appropriées.

5.1 Apports fondamentaux à la biologie de la reproduction des vanilliers

Plusieurs études ont tenté de caractériser la pollinisation des espèces du genre *Vanilla,* notamment chez les espèces américaines, où les abeilles euglossines ont été observées transportant du pollen chez *V. pompona* (Lubinsky et al. 2006; Householder et al. 2010), et seraient susceptibles de polliniser bien d'autres espèces (Soto Arenas 1999; Soto Arenas and Cameron 2003; Soto Arenas and Dressler 2010). Cependant ces abeilles sont absentes du continent africain. Les seules tentatives connues d'identification des pollinisateurs sur le continent africain ont été vaines jusqu'à présent (*V. crenulata*, Johansson en 1974 cité dans Soto Arenas and Cameron (2003)). Il s'agit donc bien, au travers de nos travaux, des premières caractérisations de pollinisateurs de vanilliers africains.

Remarquablement, chez les deux espèces étudiées, *V. roscheri* (Afrique Du Sud) et *V. humblotii* (Mayotte), des abeilles allodapines femelles ont été observées visitant les fleurs à de multiples reprises, avec notamment des captures des espèces *Allodapula variegata* et *Allodape rufogastra* avec du pollen de vanillier stocké sur les pattes postérieures ce qui suggère leur implication dans la pollinisation de *V. roscheri* (Figure 31). Les fleurs de vanilliers ont longtemps été considérées comme n'offrant pas de récompense, puisqu'elles ne produisent pas de nectar. Toutefois, d'autres récompenses doivent être considérées, comme l'arôme des fleurs et le pollen (Bembé 2004), au vu des comportements observés des insectes concernés. Le comportement de collecte du parfum des

fleurs de certains vanilliers américains par des abeilles mâles euglossines a déjà été décrit (Gigant et al. 2011). Nous rapportons ici pour la première fois dans le genre *Vanilla* que le pollen peut être considéré comme une récompense pour les insectes collecteurs de pollen, tels que les allodapines. Ces abeilles, considérées comme des pollinisateurs généralistes, sont très diversifiées en Afrique subsaharienne (Michener 1975; Schwarz et al. 2003; Chenoweth 2011), où elles pollinisent de nombreuses espèces indigènes ou introduites (Goldblatt et al. 1997; Wetschnig and Depisch 1999; Eardley and Mansell 1993; Hargreaves et al. 2010; Peter et al. 2004) et également des orchidées non-nectarifères en Australie (Van der Cingel 2001). Il est intéressant de noter que le pollen des vanilliers, comme celui d'autres groupes primitifs au sein de la famille des Orchidaceae, est de type sectile ou poudreux, très susceptible de se séparer, à l'opposé des pollens regroupés en masse pour de nombreuses lignées d'orchidées supérieures (Johnson and Edwards 2000). Les insectes collecteurs de pollen ont effectivement déjà été impliqués dans la pollinisation des taxons d'orchidées primitifs *Apostasia* (Apostasioideae) and *Cleistes* (Vanilloideae) (Gregg 1991; Dressler 1986; Freudenstein et al. 2004; Cameron et al. 1999). Le caractère sectile du pollen des vanilliers facilite la collecte du pollen par des insectes « voleurs » de pollen, tels que les allodapines, ce qui suggère que d'autres espèces de vanilliers peuvent être pollinisées par ces abeilles, en Afrique et dans d'autres régions (Asie), où elles sont présentes.

L'implication d'insectes de grandes tailles, telles que les euglossines ou les grosses abeilles charpentières sur le continent américain (Soto Arenas and Cameron 2003), a longtemps été suggérée pour la pollinisation des vanilliers (Van der Cingel 2001). Nous démontrons ici que des insectes de plus petite taille (allodapines) sont impliqués dans la pollinisation des deux vanilliers aphylles étudiés du sud-ouest de l'océan Indien. Afin de tenter de généraliser l'implication d'insectes de petite taille dans la pollinisation des vanilliers africains, nous avons comparé les tailles des colonnes des fleurs des différentes espèces décrites par Portères (1954) en fonction de leur continent d'origine ou de leur section. Nous pensons en effet qu'une réduction de la longueur de la colonne peut impliquer

une réduction de l'espace entre le gynostème et la partie basale du labelle, du fait de la forme en cornet du labelle s'élargissant vers l'extérieur, et si tel est le cas, des pollinisateurs de plus petite taille que suggérés jusqu'à présent, doivent être envisagés pour la pollinisation de certaines espèces de vanilliers. Les espèces de la section Membranaceae, aux morphologies florales particulières suggérant la pollinisation par des grosses abeilles charpentières (Soto Arenas and Cameron 2003), ont été cependant exclues. Les mesures révèlent que la taille maximale moyenne (± SE) de la colonne des vanilliers est de 27.5 (± 13.0) mm (N = 67 espèces). Les espèces foliées américaines ont les longueurs de colonne les plus élevées et significativement différentes des autres espèces (34.5 (± 12.6) mm ; N = 21 ; P = 0.020, ANOVA à un facteur). Selon notre raisonnement, ceci implique des pollinisateurs de grandes tailles, tels que les euglossines déjà identifiées dans la pollinisation de certaines espèces américaines (Lubinsky et al. 2006; Householder et al. 2010; Soto Arenas 1999; Soto Arenas and Cameron 2003; Soto Arenas and Dressler 2010). Le groupe des espèces aphylles possède les plus courtes colonnes avec 21.2 (± 6.4) mm (N = 14), et les tailles sont significativement différentes de celles des espèces foliées ($P < 0.05$). Bien que les sections Aphyllae et Foliosae soient polyphylétiques, les différences de longueur des colonnes entre vanilliers aphylles et foliés témoignent probablement d'une part, de l'origine afro-asiatique de toutes les espèces aphylles (Figure 1) (Bouetard et al. 2010), et d'autre part de la possibilité que d'autres pollinisateurs de plus petites tailles soient impliqués au moins chez les espèces aphylles, et potentiellement chez les aphylles américaines, pour lesquelles des abeilles du genre *Centris* (Anthophoridae) avaient été envisagées auparavant (Soto Arenas and Cameron 2003). Toutefois, de façon similaire, nous avons-nous-même observé d'autres pollinisateurs engagés dans la reproduction des vanilliers aphylles de l'océan Indien. En particulier, une abeille anthophorine femelle (*Amegilla* sp ou *Anthophora* sp, Pr. C. Eardley, com. pers.) a été identifiée chez *V. roscheri.* Elle appartient à la famille des Anthophoridae, comme les abeilles du genre *Centris* suggérées dans la pollinisation des vanilliers aphylles américains (Soto Arenas and Cameron 2003). Nos comparaisons suggèrent qu'il existe bien des différences notables entre les tailles de la colonne des fleurs des différents groupes de vanilliers, ce qui peut être

révélateur de l'implication de pollinisateurs de petite taille, particulièrement pour les espèces aphylles, et en complément de pollinisateurs de grande taille suggérés jusqu'à présent.

Néanmoins, nous n'excluons pas que d'autres pollinisateurs puissent intervenir dans la pollinisation des vanilliers africains notamment des aphylles. Une femelle souimanga (femelle *Nectarinia coquerelli*) a par ailleurs été identifiée chez *V. humblotii* (Figure 18). Les interactions avec les pollinisateurs différents, identifiés chez les deux espèces étudiées (autre que les abeilles allodapines), permettent de mieux comprendre les syndromes de pollinisation suggérés sur la base de la couleur des fleurs (Figure 7). En effet les espèces aux fleurs jaunes, sans récompense et sans parfum, telles que *V. humblotii*, sont susceptibles d'attirer davantage les oiseaux, alors que les espèces aux fleurs blanches sans récompense mais parfumées, telles que *V. roscheri,* attirent potentiellement plus d'insectes (Majetic et al. 2009; Schiestl et al. 2010). La pollinisation par les oiseaux implique souvent des fleurs tubulaires dans la gamme de couleur allant du rouge au jaune (Hingston and Quillan 2000), mais est souvent associée aussi aux fleurs nectarifères (Nicolson 2002). Nous avons pu constater que les deux vanilliers aphylles attiraient de nombreux insectes (fourmis, moucherons et araignées (Tomisidae)) et il est très probable que ce soit le cas pour d'autres vanilliers de l'océan Indien, et ce au moins pour l'attraction des fourmis, comme cela a déjà été montré chez de nombreux vanilliers (Peakall 1994; Lubinsky et al. 2006; Householder et al. 2010; Gigant et al. 2011). Nous suggérons donc que les espèces aux fleurs jaunes, *V. humblotii* et *V. perrieri* (présentes à Madagascar), pourraient attirer les oiseaux, non seulement par leur couleur, mais aussi par la présence d'arthropodes qui pourraient être une récompense pour les oiseaux nectarivores qui ont souvent aussi un régime insectivore. Nous avons caractérisé le souimanga *N. coquereli* à Mayotte et l'avifaune susceptible d'être attirée par *V. perrieri* à Madagascar est très diversifiée, avec de nombreux Passeriformes nectarivores présents dans différents habitats (*Cinnyris souimanga* avec trois sous-espèces décrites (Nectariniidae) et deux espèces dans chacun des genres *Neodrepanis* et *Philepitta* (Philepittidae)), dont certains ont déjà été aperçus visitant des fleurs des vanilliers aphylles (Perrier de la Bâthie 1934). En ce qui concerne les espèces aux fleurs blanches, bien que nous ayons

échoué à caractériser chimiquement les parfums des fleurs de *V. roscheri*, le parfum détecté au nez est probablement responsable du nombre élevé de visiteurs de *V. roscheri* (11 visiteurs distribués dans 4 ordres). Par ailleurs, la récente floraison de certaines accessions aphylles à fleurs blanches du CRB Vatel, met en évidence la présence de parfum, détecté au nez, ce qui suggère aussi une pollinisation par des insectes, bien qu'une analyse précise des composés émis soit nécessaire pour valider nos hypothèses. La détermination des pollinisateurs potentiels à partir des caractéristiques florales reste une tâche ardue. Des auteurs ont montré les limites du concept de syndrome de pollinisation, qu'il est nécessaire d'appréhender de façon dynamique, en considérant que les associations attendues entre caractères floraux et pollinisateurs peuvent être variables dans l'espace et dans le temps (Ollerton et al. 2009; Waser et al. 1996; Raguso 2008). Pour éventuellement mieux comprendre l'évolution des syndromes de pollinisation à l'échelle de l'océan Indien, il semble nécessaire maintenant de combiner les approches comportementale, phylogénétique et physiologique des deux parties animale et végétale en interaction.

En l'état actuel de nos connaissances, nous pouvons d'ores et déjà tenter de comparer l'efficacité des stratégies d'attraction des pollinisateurs d'un point de vue de la fructification naturelle. Le caractère « fleur blanche » paraît bien plus efficace que le caractère « fleur jaune ». En effet, l'espèce à fleur blanche (*V. roscheri*) semble attirer une plus grande diversité de pollinisateurs et a un taux de fructification bien plus élevé (26.3%) que l'espèce à fleur jaune (*V. humblotii* : 0.8%). Il s'agit d'ailleurs du taux de fructification le plus élevé jamais rapporté pour une espèce non spontanément autofertile du genre *Vanilla*. Le parfum floral particulièrement prononcé de l'espèce américaine *V. chamissonis,* a déjà été suggéré comme responsable du taux élevé de fructification observé (15%) en conditions naturelles (Macedo Reis 2000; Gigant et al. 2011). Nous pouvons donc penser qu'en plus de la couleur, le parfum émis par la fleur de *V. roscheri* est responsable de l'attraction d'un plus grand nombre de visiteurs (Majetic et al. 2009; Schiestl et al. 2010) et donc d'une plus grande efficacité de la reproduction.

L'efficacité de la pollinisation en tant que caractère lié directement à la reproduction sexuée peut être considérée comme exerçant une pression évolutive sur les espèces. Les plants de *V. humblotii* produisent en moyenne 180 fleurs par individu (34.9 fleurs par inflorescence multiplié par 5.3 inflorescences par individu dans Gigant et al. (in prep-b)) contre seulement 96 fleurs chez *V. roscheri* (13.9 fleurs par inflorescence multiplié par 7.9 inflorescences par individu dans Gigant et al. (in prep-a)), soit l'équivalent du double de fleurs produites par individu chez *V. humblotii*. Il est possible que chez *V. humblotii*, la production d'un plus grand nombre de fleurs soit une adaptation compensatoire au faible nombre de visiteurs et donc au faible taux de fructification. Selon ce raisonnement, le très faible taux de fructification de l'espèce *V. madagascariensis* en conditions naturelles à Madagascar, de l'ordre de 1%, rapporté par Perrier de la Bâthie (1934) peut être mis en relation avec le fait que l'espèce produit de 10 à 20 fleurs par inflorescence selon Portères (1954) mais 30 à 40 selon Perrier de la Bâthie (1934). A l'opposé, les espèces aphylles américaines aux forts taux de fructification, 14.5% pour *V. dilloniana* et 18.5% pour *V. barbellata* (Tremblay et al. 2005), produisent chacune seulement une douzaine de fleurs (Portères 1954). Produire d'avantage de fleurs pourrait donc être un caractère sélectionné pour une espèce faiblement visitée par les pollinisateurs.

Cependant, le faible taux de fructification de *V. humblotii* peut également être le reflet de la perte ou de la diminution des interactions avec les pollinisateurs qui est à mettre en relation avec la fragmentation extrême des populations à Mayotte dans un environnement naturel très dégradé. Des auteurs ont déjà montré les conséquences négatives de la perturbation des interactions plante/pollinisateur au sein des environnements naturels fragmentés (Steffan-Dewenter and Tscharntke 1999; Harris and Johnson 2004). Certains suggèrent même que nous sommes entrés dans une crise de la pollinisation d'envergure mondiale, d'ores et déjà montrée dans certains groupes (Segal et al. 2006), mais qui pourrait toucher des milliers de plantes à fleurs (Ingram et al. 1996) et il

s'agit probablement là, d'un des enjeux majeurs de la conservation de la biodiversité dans des environnements fragmentés (Cunningham 2000).

Ces dernières remarques tempèrent les différences d'efficacité des syndromes de pollinisation que l'on observe d'un point de vue du taux de fructification. En effet, *V. roscheri* se situe dans un environnement très conservé où la diversité biologique est exceptionnellement riche, à l'inverse des populations insulaires de *V. humblotii*, qui par définition ont une richesse spécifique inférieure au continent et qui est d'autant plus faible que nous nous situons au sein d'un environnement hautement dégradé. La poursuite de ce travail via la caractérisation de la biologie de la reproduction à l'échelle de la totalité des différentes espèces aphylles de l'océan Indien permettra d'avoir une vision plus claire et généralisable des éventuels syndromes de pollinisation associés aux couleurs des fleurs (avec deux espèces aux fleurs jaunes et cinq aux fleurs blanches). Les hypothèses formulées pourront être testées à l'issue des études sur les autres espèces aphylles et participeront à la résolution des reconstructions phylogénétiques, par une analyse cladistique utilisant les caractères discriminants à l'échelle interspécifique (présence de parfum, longueur des colonnes et nombre de fleurs par individu).

5.2 Importance de la reproduction végétative

Les études de la structuration génétique et spatiale des individus à l'échelle populationnelle ont permis pour la première fois de mesurer l'influence du mode de reproduction asexué du vanillier (« Fine-scale Spatial Genetic Structure »). Nous avons montré que la structure spatiale à petite échelle des génotypes est consécutive à l'architecture phalanx de la clonalité (Figure 19 et 20). L'agrégation des clones dans la population est liée au bouturage ou marcottage naturel des lianes, lorsque par exemple, les lianes d'un individu croissant sur différents tuteurs se rompent. Cependant, la distribution des autocorrélations génétique et spatiale dans la population (après avoir extrait les clones) montre que des individus proches dans la population (agrégés) ne sont pas nécessairement

issus de la reproduction végétative mais peuvent provenir de la germination de graines (Figure 21). Des individus proches de quelques mètres peuvent donc, tout autant être des clones que des semis, mais avec une taille maximale moyenne des clones estimée à 4.6 m dans la population de *V. humblotii* à Sohoa (Mayotte), ce qui par ailleurs justifie *a posteriori* notre méthode d'échantillonnage: la récolte à la fois des individus séparés sur différents tuteurs et l'échantillonnage des patchs d'individus agrégées tous les cinq mètres (Gigant et al. in prep-a; Gigant et al. in prep-b). Cependant, les tailles de clones sont variables, mesurées en moyenne à 19.3 m à l'échelle de Mayotte, la taille maximale entre deux clones a quant à elle été mesurée à 84 m dans la population de M'Tsamoudou, et il est donc difficile de définir un intervalle généralisable de distance entre individus qui permette d'échantillonner uniquement des génotypes différents (Gigant et al. in prep-c). La reproduction végétative peut également avoir un impact sur la reproduction sexuée. En effet, l'agrégation des individus due à la reproduction végétative, peut favoriser les autofécondations par geitonogamie, chez une espèce spontanément non-autofertile qui est aussi autocompatible et pollinisée par des abeilles, comme *V. humblotii.* A Mayotte, les effets attendus de consanguinisation (par geitonogamie) sont cependant limités par la diversité génotypique élevée des populations en moyenne (G/N = 0.72), la juxtaposition de génotypes différents dans les populations (par dispersion des graines) et le faible nombre de visiteurs des fleurs de *V. humblotii* (« Geitonogamy avoidance hypothesis » selon Dressler (1981); (Jersáková and Johnson 2006; Jersáková et al. 2006; Johnson and Nilsson 1999; Johnson et al. 2004; Smithson and Gigord 2001)).

La reproduction végétative est une stratégie de reproduction efficace permettant à la fois la colonisation du milieu et le maintien des génotypes dans le temps, ce qui peut être un avantage considérable chez une espèce qui traverse des périodes où les conditions pour la reproduction sexuée ne sont pas réunies. Il semble effectivement que les populations fragmentées et de taille réduite de *V. humblotii* ont pu maintenir leur diversité génotypique dans des conditions environnementales très défavorables où la fragmentation des habitats réduit les interactions plante-pollinisateur et entraine des réductions drastiques des effectifs des populations. Toutefois, à la

différence des arbres dont la croissance radiale de la tige permet de déterminer l'âge des individus à partir du nombre d'anneaux de croissance (dendrochronologie), les vanilliers sont des espèces longévives avec une croissance en continu des lianes à laquelle s'ajoute une multiplication végétative « à l'infini » (de Witte et al. 2011; Thomas 2002). Ces éléments compliquent la tâche du biologiste qui doit faire face à des générations chevauchantes dans la population, constituées d'individus d'âge inconnu, et ayant un taux de renouvellement indéterminé, alors que ce sont des éléments cruciaux pour le suivi évolutif de la diversité génétique dans les populations.

5.3 Les phénomènes de migration et d'isolement des populations chez les vanilliers aphylles de l'océan Indien

Les études menées sur les espèces *V. humblotii* et *V. roscheri* soulignent l'importance des phénomènes de migration dans le genre *Vanilla*, non seulement à l'échelle des temps géologiques comme cela avait été montré (Bouetard et al. 2010), mais aussi à l'échelle populationnelle (intraspécifique), comme cela est suggéré par les inférences sur la structure génétique des populations (associée ou non à leur distribution spatiale), et par l'étude des patrons d'autocorrélations spatiales et génétiques (Figure 27). Hautement significatif à moyenne distance (≤ 10km), le patron d'isolement par la distance (« Isolation-by-distance ») à Mayotte pour *V. humblotii* devient erratique pour des distances supérieures, révélant une situation complexe où la différentiation génétique (ou inversement le flux de gènes) des populations n'est pas nécessairement congruente avec leur distribution spatiale (Figure 27). Des populations relativement proches géographiquement peuvent être très différentes génétiquement sans qu'aucune barrière géographique ne puisse expliquer leur isolement génétique (Sohoa/Chiconi) (Tableau 16 et Figure 22). A l'inverse, la population insulaire de M'Bouzi possède des individus plus proches génétiquement d'une population de Grande Terre (Saziley) pourtant séparée par la mer et distante de 20 km, alors qu'un cluster génétique indépendant est formé par l'ensemble des autres individus

de M'Bouzi (Figure 24 et 26). Parallèlement, chez *V. roscheri*, nous avons pu constater l'existence de populations discontinues sur les berges du lac Sibaya (Figure 29) composées d'un unique génotype (Tableau 21). La distribution des populations suggère une certaine capacité de dispersion des graines de *V. roscheri.* Cependant, la dispersion reste limitée, car d'une part, uniquement quatre populations ont été rencontrées à Sibaya alors que des patchs de forêts similaires sont présents tout autour du lac et jusqu'au Mozambique où la plus proche population connue est à 150 km, et d'autre part, l'absence de diversité génétique révèle une absence de flux de gènes entre les populations de Sibaya et les populations septentrionales de *V. roscheri*. Si des évènements de migration à longue distance permettent d'expliquer la distribution actuelle à la fois de *V. humblotii* et de *V. roscheri* dans des territoires isolées et parfois insulaires (les populations de *V. humblotii* sur les îlots de M'Bouzi et Moya), il s'agit probablement d'évènements rares et stochastiques. En effet, la déhiscence des fruits des deux espèces (obs. pers.) libère des graines de petites tailles de moins d'un millimètre qui sont probablement facilement dispersées, comme pour de nombreuses orchidées (Dressler 1981). Chez *V. humblotii,* l'influence de la gravité et du vent explique probablement la dispersion des graines à courte distance qui se traduit par l'absence d'autocorrélation génétique et spatiale à petite distance (après exclusion des clones). En revanche, les migrations à grande distance nécessitent de faire appel à des facteurs abiotiques d'envergure régionale et/ou biotiques par l'intervention de disperseurs naturels des graines de vanilliers dans l'océan Indien. La zone du sud-ouest de l'océan Indien est soumise à différentes influences atmosphériques d'intensités variables. Mayotte, par exemple dans la zone de convergence inter-tropicale, subit non seulement l'influence des moussons, un vent du nord chaud et humide parfois violent qui provient des zones équatoriales, mais aussi de perturbations, qui en fonction de leur intensité prennent le nom de dépression tropicale ou de cyclone. Ces évènements de grande envergure, d'un diamètre atteignant 1000 km selon les systèmes dépressionnaires, peuvent être impliqués dans la distribution de *V. humblotii* à l'échelle de Mayotte, mais également plus largement à l'échelle de l'archipel des Comores. L'influence des moussons africaines ou des moussons subtropicales en Afrique Du Sud (Jianping and Qingcun 2003),

accompagnées parfois de vents violents, a pu entrainer la migration des graines de *V. roscheri* depuis le Mozambique ou la Tanzanie vers l'Afrique Du Sud. Dans le groupe des orchidées angraecoïdes de l'océan Indien, les études phylogénétiques ont ainsi montré les multiples évènements de colonisation probablement liés aux évènements cycloniques et à l'origine de la radiation de ces orchidées au sein des Mascareignes à partir d'ancêtres malgaches (Micheneau et al. 2008). A Mayotte, les analyses phytogéographiques ont montré que plusieurs vagues successives de colonisation ont permis l'établissement de la flore à partir d'ancêtres malgache et africain (Pascal et al. 2001). Ces différents exemples suggèrent l'importance des évènements climatologiques dans la compréhension des mécanismes de dispersion des populations végétales à l'échelle de la région du sud-ouest de l'océan Indien.

Les facteurs biotiques peuvent également intervenir dans la dispersion des vanilliers. Ces évènements semblent néanmoins rester occasionnels car la consommation de fruits n'a été observée qu'une seule fois dans chacune des espèces étudiées (obs. pers.) tout comme rarement chez l'espèce américaine *V. pompona* subs. g*randiflora* au Pérou (Householder et al. 2010). Cependant, des preuves de dispersion par des chauves-souris ont été détectées chez les deux espèces américaines, *V. pompona* et *V. insignis* (Soto Arenas and Dressler 2010). Il existe d'ailleurs, dans le sud-est africain et dans l'océan Indien, une très grande diversité de chauves-souris, notamment de chauves-souris fruticoles avec quatre genres présents, *Roussetus* (2 espèces)*, Pteropus* (9 espèces), *Eidolon* (1 espèce) et *Epomophorus* (1 espèce) (Carroll and Feistner 1996; O'Brien 2011). Dans l'ensemble, le régime de ces espèces est assez mal connu (Carroll and Feistner 1996). Néanmoins, nous savons que le genre *Pteropus,* le plus diversifié dans la zone, consomme une large variété de fruits, de fleurs et de feuilles et comprend notamment l'espèce *P. seychellensis comorensis,* connue pour disperser les graines de nombreuses espèces végétales indigènes (O'Brien 2011; Carroll and Feistner 1996). Les vols pour la recherche de nourriture se limitent de 10 à 40 kms du perchoir en fonction des espèces étudiées (O'Brien 2011), mais si l'on tient compte de leur capacité de rétention des graines dans leur système digestif, les distances de dispersion de petites graines peuvent dépasser plusieurs centaines

de kilomètres (Shilton et al. 1999). Par ailleurs, Goodman et al. (2010) ont souligné récemment la faiblesse des barrières géographiques entre les îles de l'archipel des Comores avec l'existence probable d'échanges entre populations de certaines espèces de chauve-souris. Dans les Mascareignes, la roussette noire *Pteropus niger*, qui avait disparu à La Réunion depuis le XIXème siècle, y a été redécouverte récemment (entre 2000 et 2007), et c'est probablement lors d'un évènement cyclonique que des individus ont migré accidentellement à partir de l'île Maurice, située à environ 200 km à l'est de La Réunion. Dans le sud-est africain, la mousson a aussi probablement été impliquée dans la dispersion et la radiation de certains groupes de chauves-souris asiatiques dans la zone du sud-ouest de l'océan Indien (Meirte (1984) cité dans O'Brien (2011).

En résumé, de nombreux vecteurs peuvent être suggérés dans la dispersion des vanilliers dans l'océan Indien. Nous soupçonnons que leur rareté et leur imprévisibilité sont des caractères majeurs qui peuvent être responsables à la fois de la parenté inattendue détectée entre certaines populations, et de la différentiation rencontrée dans d'autres, pourtant proches géographiquement.

La variabilité des flux de gène dans les populations de *V. humblotii* à Mayotte (mesurée en nombre de migrants (*N*m) : $0.575 \leq Nm \leq 1.790$) et l'absence vraisemblable de flux de gènes chez *V. roscheri* dans le sud-est africain, suggèrent que ces populations de vanilliers peuvent être soumises, en fonction de l'intensité des flux de gènes entre populations, à des contraintes évolutives différentes, telles que la dérive génétique et l'adaptation locale liée à la sélection et qui peuvent engendrer de la spéciation (Tremblay and Ackerman 2003). Effectivement, dans le cas où le flux de gène entre populations est entièrement rompu, les influences des facteurs écologique et génétique et leurs interactions peuvent être des sources d'apparition de nouvelles formes conduisant à l'apparition de nouvelles espèces. Rigoureusement, la spéciation s'accompagne d'un isolement reproductif qui peut être génétique (remaniements chromosomiques, polyploïdisation) et/ou écologique (période de floraison, séparation géographique). Cependant, l'expérience a déjà montré que les barrières interspécifiques peuvent être transgressées chez les vanilliers, non seulement par l'intervention de l'homme (hybridations interspécifiques, (Bourriquet 1954; Childers and Cibes 1948)) mais aussi en

conditions naturelles (Nielsen 2000; Nielsen and Siegismund 1999). La variabilité et la stochasticité des flux de gènes entre populations, et l'absence de barrières reproductives fortes entre vanilliers peuvent nous aider à appréhender la complexité des relations interspécifiques entre vanilliers de l'océan Indien, et notamment entre espèces en sympatrie. A Madagascar par exemple, où différentes espèces de vanilliers à fleur blanche sont en sympatrie, il est probable que des hybridations naturelles se produisent, comme cela a déjà été montrées chez des espèces aphylles américaines en sympatrie à Puerto Rico (Nielsen 2000; Nielsen and Siegismund 1999). Perrier de la Bâthie (1934) signale d'ailleurs que les fleurs des vanilliers aphylles malgaches, sans préciser les espèces concernées, sont visitées par les mêmes oiseaux-mouches (*Nectarinia* sp. i.e. probablement du genre *Cinnyris* actuel), ce qui peut engendrer des hybridations naturelles interspécifiques. Si l'identification du même groupe d'abeille sauvage (les allodapines) chez les deux espèces étudiées dans cette thèse (pour lequel le dimorphisme floral est très marqué), était confirmée pour les autres vanilliers de la zone l'océan Indien, des hybridations naturelles pourraient être suggérées plus largement à l'échelle de la zone. Ainsi, la diversité des morphologies spécifiques à l'échelle de Madagascar, mais également du sud-ouest de l'océan Indien (Figure 7), pourrait être interprétée comme un degré d'isolement variable des populations de la zone, si l'on tient compte de l'importance des phénomènes de migration à longue distance des graines, et des possibilités d'hybridations naturelles issues de mouvements de pollen entre populations d'espèces en sympatrie. Cameron (2011) a d'ailleurs suggéré que certaines espèces de vanilliers aphylles à fleur blanche de l'océan Indien pouvaient représenter les variations morphologiques d'une seule et même espèce. Nous avons nous-mêmes soulevé la difficulté de différentier phylogénétiquement certaines espèces aphylles de la zone (Gigant 2008). Il est probable que les populations soient soumises à des contraintes écologiques et/ou des influences génétiques différentes, qui provoquent l'émergence de populations aux morphologies sensiblement différentes des populations d'origine, et potentiellement bien adaptées aux conditions locales, mais partageant un fond morphogénétique commun en interaction dynamique avec l'environnement.

5.4 L'approche intégrative de la biologie de la reproduction et de la génétique des populations et la complémentarité des modèles

L'étude de la biologie de la reproduction et de la diversité génétique des deux vanilliers aphylles *V. humblotii* et *V. roscheri* a révélé l'importance et la complémentarité des approches pour améliorer nos connaissances et optimiser la conservation des espèces sauvages du genre *Vanilla.* En effet, *V. humblotii* montre un très faible taux de fructification naturelle (0.8%) associé à une diversité génétique élevée et des taux d'hétérozygotie à l'équilibre panmictique à l'échelle de Mayotte et dans la quasi-totalité des populations étudiées. L'espèce *V. roscheri* montre, quant à elle, le taux de fructification naturelle le plus élevé pour une espèce du genre *Vanilla* non-autofertile spontanément pourtant associé à une diversité génétique nulle, poussée à l'extrême, puisque nous rapportons un cas unique de perte totale de diversité allélique, où tous les marqueurs sont homozygotes pour l'ensemble des individus des populations du lac Sibaya.

Ces résultats mettent en évidence deux situations clairement paradoxales. Nous pouvions nous attendre en effet, d'une part, à ce que *V. humblotii* distribuée au sein de fragments forestiers extrêmement réduits et isolés pour la plupart, exprime une très forte consanguinisation et d'autre part, à ce que *V. roscheri,* ayant un taux de fructification exceptionnellement élevé, montre une diversité génétique importante. La complémentarité des approches écologique et génétique prend tout son sens, au regard de la réalité écologique de la situation de *V. humblotii* incapable de se reproduire sexuellement, et de la réalité génétique de *V. roscheri,* qui malgré un taux de fructification élevé, est incapable de générer de la diversité en l'absence de variabilité allélique à Sibaya. Les deux espèces doivent donc faire face à des contraintes écologique ou génétique qui débouchent dans les deux cas sur une impasse évolutive par l'incapacité à générer de la diversité, pour s'adapter à la perturbation des milieux naturels.

5.5 Implications pour la conservation des espèces étudiées

La population d'une espèce en danger localement (*V. humblotii* dans certains fragments forestiers; ou *V. roscheri* en Afrique Du Sud), mais potentiellement présente ailleurs et/ou sous d'autres formes, mérite-t-elle des mesures particulières de conservation ? Nous pensons que oui, car les populations en périphérie de distribution d'une espèce sont des sources potentielles de diversité génétique, souvent unique. Nous l'avons montré chez *V. roscheri*, et dans d'autres populations fragmentées de *V. humblotii* (notamment insulaires ou parfois réduites à moins de 10 individus), où se trouvent des phénotypes potentiellement bien adaptés aux conditions environnementales de la distribution limite (Stöcklin et al. 2009; Eckhart et al. 2011; Larcher et al. 2010). Ces populations fragmentées ou marginales, doivent donc faire l'objet de mesures appropriées de conservation, car elles sont des sources de variabilité essentielles pour la survie des espèces à long terme.

Les résultats obtenus montrent qu'il est important de prendre des mesures de conservation adaptées à la situation de chaque espèce. Pour *V. humblotii*, bien que la majorité des populations ait semblé conserver une importante diversité génotypique, résistant à la réduction des tailles de populations par la présence de la reproduction végétative, il est inquiétant de constater une absence quasi-totale de reproduction sexuée. La récente fragmentation des environnements naturels, liée à l'augmentation des pressions anthropiques à Mayotte (Trouillard et al. 2009; Audru et al. 2010), est probablement responsable de la perte ou de la diminution des interactions avec les pollinisateurs, et du faible niveau de reproduction sexuée. Ce sont donc les conséquences écologiques des activités humaines sur la reproduction sexuée de *V. humblotii* à Mayotte, qui sont préoccupantes actuellement. La viabilité à long terme des espèces dépend de la reproduction sexuée, qui permet le maintien des potentialités évolutives afin de faire face aux modifications environnementales (Ouborg et al. 2006). La population de Sohoa, où l'on a détecté la majeure partie des évènements de reproduction sexuée, semble être une des seules offrant les conditions suffisantes et nécessaires pour permettre le maintien de populations naturelles de *V. humblotii*, et nous pensons que les

efforts de conservation *in situ* doivent se concentrer sur cette population. Ce fragment de forêt, le plus important et un des deux seuls fragments de forêts mésophiles de Mayotte, est donc une priorité majeure pour le maintien de la variabilité des habitats naturels de *V. humblotii* et probablement d'autres espèces végétales et animales associées à ce type de forêt. Cependant, le signe de consanguinisation détecté pour un des marqueurs génétiques, révèle probablement un excès de croisements entre individus proches géographiquement et/ou génétiquement (à mettre en relation avec la distribution phalanx de la clonalité, la pollinisation par des abeilles et l'absence de système d'auto-incompatibilité à la reproduction), et donc le risque d'une érosion génétique de la population par la fixation aléatoire de certains allèles (dérive génétique). La population nécessite donc un suivi de son évolution génétique dans le temps. Si une dérive génétique ou une consanguinisation venaient à s'installer progressivement dans la population, par l'absence de flux de gènes avec d'autres de population de Mayotte et l'importance de la clonalité, des croisements manuels à l'intérieur de la population entre différents génotypes pourraient s'avérer nécessaire pour favoriser le maintien de la diversité génotypique dans Sohoa. A l'échelle de Mayotte, de toute évidence, le maintien de la diversité totale de *V. humblotii* nécessite de tenir compte aussi des petites populations encore présentes dans les fragments de forêt naturelle, qui sont des sources de diversité génétique originale, avec notamment l'identification de certains allèles strictement présents dans ces populations (Tableau 14).

Si les menaces d'une perte de diversité génétique à Sohoa sont présentes, elles ne montreront probablement leurs effets que dans le futur en l'absence de flux de gène. En revanche, la population de l'îlot M'Bouzi (82 ha), dans la seule Réserve Nationale Terrestre de Mayotte, pourrait dès maintenant faire l'objet de mesures de conservation particulières *in situ.* L'urgence réside dans le fait que l'ensemble des individus de la population est infecté par la cochenille *Conchaspis angraeci*, et de nombreux individus sont dans un état de dépérissement avancé dont les fragments de lianes infectés mesurent moins d'un mètre (obs. pers. et Figure 11). De plus, la prédation des fleurs (et des bourgeons floraux) de *V. humblotii* sur l'îlot, probablement causée par les rongeurs introduits et lié

au manque de ressource en eau de l'îlot, diminue nécessairement la capacité à fructifier des individus. Les populations de *V. humblotii* à M'Bouzi pourraient donc être sujettes à un programme de conservation approprié, consistant non seulement à réintroduire de la diversité génétique dans la population, mais également à contrôler ou à éradiquer les populations de nuisibles. Ce programme de conservation de *V. humblotii* nécessite une collaboration approfondie entre les biologistes de la conservation et les agents de la conservation, comme suggéré par Donlan et al. (2003). Un programme de réintroduction de la diversité sur l'îlot M'Bouzi permettrait d'augmenter son potentiel évolutif. L'augmentation de la diversité génétique peut être envisagé selon trois procédés : (i) par des croisements manuels entre génotypes différents de la population (tel que proposé pour la conservation de la population de Sohoa avec éventuellement en plus, un suivi rigoureux des inflorescences si la pression de prédation est très forte), (ii) par l'implantation directe d'individus provenant de populations différentes, ou (iii) par un croisement *ex situ* avec des individus de différentes populations. Cependant, les croisements entre individus de différentes populations nécessitent d'utiliser des populations les plus proches génétiquement et/ou écologiquement afin de limiter l'expression d'une éventuelle « outbreeding depression » (le pendant négatif du bénéfice attendu lors de flux de gènes entre populations génétiquement différenciées), qui pourrait diminuer la viabilité de la population. L'avantage d'un programme de reproduction *ex situ*, avant une réintroduction de la descendance dans la population naturelle, est que la descendance peut être évaluée en termes de viabilité et ainsi garantir le succès de l'établissement des individus introduits. En l'état actuel de nos connaissances sur la différentiation génétique et écologique des populations de *V. humblotii* à Mayotte, les populations des sites de Choungui et de Saziley (non fortement différentiés génétiquement et aux conditions écologiques similaires), pour lesquels certains individus sont d'ores et déjà disponibles au CRB Vatel, sont probablement les plus aptes à être intégrées dans un programme de reproduction impliquant les individus de M'Bouzi. La réussite des étapes de réintroduction et du maintien de la population nécessite cependant un suivi des individus et doit tenir compte en parallèle des rongeurs introduits sur l'îlot. Il est donc nécessaire d'impliquer les

agents de la conservation dans la lutte ou le contrôle des populations de rongeur. Dans leur revue bibliographique, Howald et al. (2007) ont montré l'efficacité et la nécessité d'éradiquer les rongeurs introduits des îlots de petite taille (< 100 ha). Par ailleurs, le caractère protégé de l'îlot (Réserve Naturelle Nationale), qui devrait probablement permettre un retour progressif de la végétation naturelle et limiter l'introduction de nouvelles espèces invasives, en fait un bon modèle de banque génétique forestière (« Forest gene bank ») comme proposée par Uma Shaanker and Ganeshaiah (1997). Car comme nous l'envisageons pour *V. humblotii* sur l'îlot M'Bouzi, leur concept consiste à introduire et maintenir dans des environnements naturels le matériel génétique de différentes sources.

En Afrique Du Sud, les populations de *V. roscheri* ont un taux de fructification élevé et semblent en mesure de se maintenir en l'état actuel des conditions environnementales. Bien que nous ayons rapporté un cas unique de consanguinisation extrême (homozygotie généralisée à tous les loci), aucun signe d'une dépression de consanguinité n'a en effet été détecté. Il est probable que l'unique génotype présent à Sibaya, ait purgé son fardeau génétique (Reed et al. 2003) et qu'il soit particulièrement bien adapté aux conditions de Sibaya en limite d'aire de répartition de l'espèce (Eckhart et al. 2011). Au regard de l'originalité à la fois de la composition génétique et de la distribution de l'espèce, la protection *in situ* des populations de Sibaya apparaît nécessaire. La protection *ex situ* du génotype représentatif est aussi indispensable et fort simple à mettre en œuvre. Toutefois, dans un environnement en constante modification, l'absence de diversité génétique limite considérablement les potentialités évolutives de *V. roscheri* pour faire face aux menaces directes sur l'habitat de *V. roscheri,* telles que la compétition avec les espèces invasives, la déforestation et l'aridification des forêts tropicales (Le Treut and Jancovici 2004; Smith et al. 2008; Smith and Leader-Williams 2006; Von Maltitz et al. 2003). Il serait donc envisageable de réintroduire de la diversité génétique dans la population de Sibaya à partir des populations septentrionales (ou de l'accession CR0810, issue de Zanzibar). Dans ce cas, les risques liés à l'introduction de nouveaux

allèles potentiellement délétères dans la population de Sibaya, sont très importants et menacent de bouleverser un équilibre des populations par l'expression d'une forte « outbreeding depression ». Il est en effet probable que les populations de Sibaya soient isolées génétiquement des autres populations septentrionales depuis fort longtemps au regard de l'absence de diversité génétique. C'est pourquoi, il semble préférable de réaliser des croisements *ex situ* entre différents génotypes de *V. roscheri*, de tester leur viabilité et la fertilité de la descendance avant d'engager des réintroductions dans Sibaya (Volis et al. 2009). Cependant, ce type de programme est onéreux et dépendra aussi de la diversité qui sera mise en évidence dans les autres sites où l'espèce est présente.

Les approches de la conservation que nous suggérons pour les deux vanilliers aphylles étudiés sont en accord avec le concept de conservation *quasi in situ* proposé par Volis and Blecher (2010); (Volis et al. 2009) avec la prise en compte (i) d'un échantillonnage représentatif des populations ; (ii) du maintien des populations en collection et (iii) d'une utilisation de ces ressources génétiques dans l'objectif d'une conservation *in situ* des populations. L'échantillonnage réalisé au sein des populations naturelles a permis de décrire fidèlement la diversité génétique des populations des espèces étudiées et de rendre compte de la plasticité écologique de ces espèces. Les études de la biologie de la reproduction et de la génétique des populations ont permis de caractériser ces populations naturelles. Dans l'optique de maintenir la diversité des populations naturelles qui sont précieuses pour les programmes de conservation et/ou de reproduction des espèces, il convient de maintenir *ex situ* sous différentes formes le matériel échantillonné (*in vivo* ou en culture *in vitro*, extrait d'ADN, et matériel cryogénisé), mais cette entreprise est onéreuse. Néanmoins, au-delà la conservation de la biodiversité, ces opérations peuvent se justifier par l'intérêt que peut représenter les espèces aphylles pour l'amélioration de l'espèce cultivée, *V. planifolia*, en tant que sources de caractères agronomiques. Une core-collection pourra être établie, en ne conservant *in vivo* qu'un sous-ensemble des populations maximisant la représentativité génétique et la variabilité écologique

des populations ce qui permettra de répondre efficacement au besoin de conservation des populations naturelles.

5.6 Perspectives de recherche

Les travaux que nous avons accomplis au cours de cette thèse ont permis des avancées majeures dans la compréhension de l'influence des modes de reproduction (sexuée et asexuée) dans le maintien des populations naturelles des vanilliers sauvages étudiés. Par ailleurs, nous avons montré l'importance de l'approche intégrative de la biologie de la reproduction et de la génétique des populations pour formuler des propositions de conservation appropriées.

En premier lieu, les résultats que nous avons obtenus nous permettrons d'appuyer une évaluation IUCN des deux espèces étudiées, en vue d'un classement rationalisé de ces espèces pour une reconnaissance internationale de la nécessité de la protection des populations identifiées. Ces outils de la conservation serviront de support au plan de conservation en cours de développement par le Conservatoire Botanique National de Mascarin pour l'espèce *V. humblotii* à Mayotte mais également à la volonté des autorités du Ezemvelo KwaZulu Natal Wildlife d'étendre la zone de protection déjà existante (cantonnée actuellement aux parties immergées et à la bande de sable séparant le lac de l'océan Indien), aux berges et aux forêts tropicales sèches à proximité du lac Sibaya.

Nos études mettent aussi en perspective la nécessité de poursuivre l'échantillonnage des populations de chacune des espèces dans de nouveaux sites :

- A l'échelle de l'ensemble de l'archipel des Comores pour *V. humblotii*, sur les îles de Grande Comore et d'Anjouan en premier lieu, où des échantillons ont déjà été récoltés dans le passé, puis sur l'île de Mohéli, pour laquelle les données d'inventaires floristiques sont limitées. Les prospections pourront être étendues également au nord-est de Madagascar où l'espèce a été signalée récemment par Cribb et al. (2009)

- A l'échelle du littoral sud-est africain pour *V. roscheri*, dans les populations supposées du Kenya au Mozambique en passant par la Tanzanie et les îles Pemba et Zanzibar où des collectes ont été menées (Da Silva et al. 2004; La Croix and Cribb 1995; Krupko et al. 1954; Beentje 1990).

De plus, il semble nécessaire de compléter l'échantillonnage des espèces aphylles de l'océan Indien en ciblant les espèces malgaches (*V. madagascariensis*, *V. decaryana, V. montagnacii* et *V. perrieri*) et l'espèce seychelloise (*V. phalaenopsis*) afin de mieux comprendre l'évolution des interactions plante-pollinisateur dans l'océan Indien, de rendre compte de toute la diversité des formes en présence, d'identifier les niveaux de diversité génétique des populations et de clarifier les relations phylogénétiques interspécifiques des vanilliers de l'océan Indien. Les microsatellites développés lors de cette thèse seront un apport précieux dans ce cadre, et leur utilisation devra être complétée par l'utilisation de marqueurs microsatellites chloroplastiques tout comme par la poursuite des analyses phylogéographiques en ciblant des régions suffisamment variables telles que la région *trn*H-*psb*A ou les ITS nucléaires.

Par ailleurs, le séquençage en cours du génome de *V. planifolia* et de *V. bahiana,* un projet en collaboration avec l'Université de Davis en Californie et le JCVI (J. Craig Venter Institute, Etats-Unis), permettra de développer de nouveaux marqueurs à l'échelle génomique, utilisables pour résoudre les relations phylogénétiques des espèces de l'océan Indien, mais aussi pour approfondir les études de la diversité génétique des populations. Les potentialités du développement des techniques de « séquençage de nouvelle génération » permettent effectivement d'appréhender la diversité populationnelle des espèces à une nouvelle échelle, par une approche génomique de la conservation qui offre la possibilité d'étudier les interactions du génome avec l'environnement par (i) l'utilisation simultanée de nombreux marqueurs génétiques, (ii) la distinction des marqueurs neutres de ceux soumis à sélection et (iii) une estimation fidèle de l'histoire évolutive et de la démographie des populations par exclusion des marqueurs non-neutres (Ouborg et al. 2010). Identifier les régions du génome soumises à sélection permettra de différentier notamment la dérive génétique des effets de

la sélection naturelle. Ceci ouvre la voie à l'étude des effets de la qualité de l'habitat des populations naturelles et donc de l'influence des facteurs sur la viabilité des populations par une intégration des menaces génétiques et environnementales.

Par ailleurs, d'autres approches en « omiques » (transcriptomique et métabolomique) sont amenées à se développer et sont effectivement des étapes prometteuses dans la compréhension des mécanismes sous-jacents à l'adaptation et au maintien de la diversité des espèces dans leur environnement naturel.

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Reproductive biology and genetic and spatial diversity of two species of *Vanilla* genus (Orchidaceae) from the South West Indian Ocean: *V. humblotii* and *V. roscheri.* Implications for their conservation.

To deal with environmental upheavals, the development of approaches combining reproductive biology and genetic diversity of plant species is crucial in order to improve and optimize biodiversity conservation. The South-West Indian Ocean comprises a monophyletic group of seven leafless species of *Vanilla* genus Plum Ex Miller (Orchidaceae, Vanilloideae), including *V. humblotii* Rchb. f. and *V. roscheri* Rchb. f. endemic to the Comoros Archipelago and southeastern Africa, respectively. Their vestigial leaves, scale-like, confer an adaptation to dry tropical forests, but these species are also found in mesophilous forests. In a context where the cultivated *V. planifolia* G. Jackson species, source of the vanilla pod, is particularly vulnerable to biotic and abiotic threats due to a reduced genetic diversity, the ecological plasticity of leafless vanilla is a major character potentially exploitable to improve vanilla crop.

Prospecting missions allowed the identification and the sampling of wild populations of *V. humblotii* in Mayotte (Comoros Archipelago) and *V. roscheri* on the banks of Lake Sibaya in KwaZulu Natal (South Africa). A study of the reproductive biology, associating the evaluation of incompatibility and mating systems and pollinator identifications, was performed in the two species. In parallel, microsatellite markers were developed from these species to characterize the population genetic diversity, the influence of the mating systems on the spatial and genetic structure of the species at different scales.

In Mayotte Island, the fragmentation of the wild populations of *V. humblotii* resulted in the loss of allelic diversity, which was detected in several small populations. Nevertheless, the long-lived character of *V. humblotii,* driven by vegetative reproduction, allowed the maintenance of a high genotypic diversity (G/N = 0.88) and limited the deleterious impacts of drastic reductions of population sizes. Although the phalanx architecture of the clonality was responsible for the genetic and spatial autocorrelations observed at small scale (<10m), the higher seed dispersal than pollen dispersal limited the possibilities of inbreeding by geitonogamy. However, the in-depth study of reproduction revealed a low level of natural fruit set (0.8%) and the quasi-total loss of plant-pollinator interactions, even if a female allodapine bee, *Allodape obscuripennis* Strand (Apidae, Xylocopinae, Allodapini), and a female souimanga, *Nectarinia coquerelli* (Passeriformes, Nectariniidae), were observed visiting the flowers of *V. humblotii.*

In South Africa, the genetic analysis of the populations of *V. roscheri* revealed an unprecedented case of total loss of genetic diversity and total homozygosity in all the microsatellite loci tested. In the species range-edge distribution in South Africa, the populations have probably suffered from a strong bottleneck (by migration or population size reduction) followed by strong inbreeding. But, the highest fruit set (26.3%), among the non-spontaneous self-fertile species in the *Vanilla* genus, was associated with pollen movements and two female allodapine bees (*Allodapula variegata* Smith and *Allodape rufogastra* Lepeletier et Serville (Apidae, Xylocopinae, Allodapini)) and one female anthophorine bee (Apidae, Apinae, Anthophorini *sp*) were characterized as the main pollinators of *V. roscheri.*

The studied models highlight the complementary nature of the genetic and ecological approaches employed. On one hand, the reproductive studies allowed to evaluate the deleterious effects of habitat fragmentation in the wild *V. humblotii* populations from Mayotte and on the other hand, the genetic studies were crucial to measure the isolation and genetic impoverishment of *V. roscheri* populations from South Africa. The two situations emphasize the low evolutionary potential of the species to deal with environmental changes and therefore, the wild populations sustainability is jeopardized by ecological or genetic threats. In the aim to optimize the management of these wild *Vanilla* species from the South-West Indian Ocean area, *in situ* and *ex situ* conservation guidelines are proposed.

Keywords : Conservation, reproductive biology, genetic diversity, Orchidaceae, anthropogenic threats, pollinator, leaflessness, *Vanilla humblotii*, *Vanilla roscheri.*

Biologie de la reproduction et diversité génétique et spatiale de deux espèces du genre *Vanilla* (Orchidaceae) du sud-ouest de l'océan Indien : *V. humblotii* et *V. roscheri.* Implications pour leur conservation.

Face aux menaces des grands bouleversements environnementaux, le développement des approches combinées de la biologie de la reproduction et de la diversité génétique des espèces végétales s'avère être essentiel pour améliorer et optimiser la conservation de la biodiversité.

Le sud-ouest de l'océan Indien abrite un groupe monophylétique de sept espèces aphylles du genre *Vanilla* Plum Ex Miller (Orchidaceae, Vanilloideae), dont *V. humblotii* Rchb. f. et *V. roscheri* Rchb. f. endémiques de l'archipel des Comores et du sud-est africain respectivement. Leurs feuilles vestigiales, semblables à des écailles, leurs confèrent une adaptation aux forêts tropicales sèches mais elles peuvent se retrouver également en forêt tropicale mésophile. Dans un contexte où l'espèce cultivée du genre *Vanilla, V. planifolia* G. Jackson à l'origine de la gousse de vanille, est particulièrement vulnérable aux agressions biotique et abiotique à cause d'une base génétique extrêmement réduite dans les cultures, la plasticité écologique des vanilliers aphylles est un caractère majeur potentiellement exploitable pour améliorer l'espèce cultivée.

Des missions de prospections ont permis d'identifier et d'échantillonner les populations naturelles de *V. humblotii* à Mayotte (Archipel Des Comores) et de *V. roscheri* sur les berges du lac Sibaya dans le KwaZulu Natal (Afrique Du Sud). Une étude de leur biologie de la reproduction, associant une évaluation des systèmes d'incompatibilité, des régimes de reproduction et une identification des pollinisateurs a été réalisée chez les deux espèces. En parallèle, des marqueurs microsatellites ont été développés à partir de ces espèces pour évaluer la diversité génétique de leurs populations, l'influence des modes et des régimes de reproduction sur la structuration génétique et spatiale des espèces à différentes échelles.

A Mayotte, la fragmentation des populations naturelles de *V. humblotii* a entrainé la perte de diversité allélique mesurée dans de nombreuses populations de taille réduite. Néanmoins, le caractère longévif de *V. humblotii,* favorisé par la reproduction végétative, a permis de maintenir une diversité génotypique élevée (G/N = 0.88) et de limiter les impacts délétères de la réduction drastique des tailles de population. Bien que l'architecture phalanx de la clonalité soit majoritairement responsable des autocorrélations génétique et spatiale à petite distance (<10m), la dispersion des graines supérieure à la dispersion du pollen limite les possibilités de croisements consanguins par geitonogamie. Cependant, l'étude approfondie de la reproduction a révélé un faible taux de fructification naturelle (0.8%) et la perte quasi-totale des interactions plante-pollinisateurs, bien qu'une femelle abeille allodapine, *Allodape obscuripennis* Strand (Apidae, Xylocopinae, Allodapini), et une femelle souimanga, *Nectarinia coquerelli* (Passeriformes, Nectariniidae), aient été observées comme visiteurs des fleurs de *V. humblotii.*

En Afrique Du Sud, l'analyse génétique des populations de *V. roscheri* révèle un cas sans précédent de perte totale de diversité génétique avec une homozygotie généralisée à l'ensemble des marqueurs microsatellites. En limite d'aire de répartition de l'espèce en Afrique Du Sud, les populations ont probablement subi un fort goulot d'étranglement (par migration ou fragmentation de population) suivie d'une importante consanguinisation. En revanche, le taux de fructification moyen est le plus élevé (26.3%) jamais rapporté pour une espèce allogame du genre *Vanilla,* avec de nombreux visiteurs des fleurs associés à des mouvements de pollen, dont notamment, deux abeilles allodapines femelles (*Allodapula variegata* Smith et *Allodape rufogastra* Lepeletier et Serville (Apidae, Xylocopinae, Allodapini)), et une abeille anthophorine femelle (Apidae, Apinae, Anthophorini *sp*), caractérisées comme étant les principaux pollinisateurs de *V. roscheri.*

Les modèles étudiés soulignent la complémentarité des approches génétique et écologique utilisées. D'un côté, les études de la reproduction ont permis de mesurer les impacts délétères de la fragmentation des habitats sur les populations naturelles de *V. humblotii* à Mayotte, et de l'autre, les études génétiques se sont avérées essentielles pour évaluer l'isolement et l'appauvrissement génétique des populations de *V. roscheri* en Afrique Du Sud. Les deux situations mettent en évidence le faible potentiel évolutif de ces espèces pour faire face aux modifications environnementales. Des menaces majeures d'ordre écologique ou génétique pèsent donc sur la viabilité des populations naturelles. Des mesures de conservation *in situ* et *ex situ* sont alors proposées pour optimiser la conservation de ces vanilliers sauvages du sud-ouest de l'océan Indien.

Mots clés : conservation, biologie de la reproduction, diversité génétique, Orchidaceae, menaces anthropiques, pollinisateur, Aphyllie, *Vanilla humblotii*, *Vanilla roscheri.*